A FROZEN BLOOD BANK

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Summary

Stimulated by the outbreak of type B hepatitis in the renal dialysis and transplant unit in which both patients and staff died, a frozen blood bank has been established in the Edinburgh Blood Transfusion Service. The method adopted in freezing utilizes a low concentration of glycerol, a penetrating additive, to protect the red blood cells against the damaging effects of freezing. The glycerolized erythrocytes, placed in aluminium cans, are frozen rapidly in liquid nitrogen and stored in the vapour phase for as long as required.

Only group "O" Rh-positive and group "O" Rh-negative blood is used for freezing and typing for other blood group antigens is performed as far as the availability of antisera permits.

Deglycerolization is accomplished by serial centrifugal "batch washing" either manually in five-tailed Tuta bag or automatically in the IBM 2991 Cell Processor. A comparative study between both methods showed that automatic washing in the IBM offers the following advantages.

1. Being an automatic process it saves both time and effort of the operator.
2. It gives higher red cell recoveries.
3. It washes better as evidenced by the residual glycerol and sorbitol content of the supernatant fluid.
4. As the number of open manipulations are limited, blood processed by the IBM 2991 is less liable to bacterial contamination and thus can be stored at $4^{o}C$ for longer periods than that processed manually.

Immediately after processing and resuspension in isotomic saline the red blood cell have an acceptable level of intracellular potassium, 2,3 DPG and ATP content. However, on further storage at $4^{o}C$ the cells were unstable and showed a high rate of autohaemolysis evidenced by the rapidly increasing levels of extracellular potassium and free haemoglobin.

After exhaustive studies we found that the reconstitution of the processed erythrocytes in an equal volume of a medium made up from isotomic saline and 70 ml acid-citrate-dextrose solution remarkably improved the post-thaw stability. Thus, there was a four fold reduction in the supernatant free haemoglobin whereas the extracellular potassium was reduced to half of its concentration in isotonic saline alone. This effect is due to the low pH and citrate content of the ACD solution.

Bacteriological studies on blood processed in the IBM 2991 and reconstituted in saline-ACD medium showed that this blood was sterile for at least ten days of post-thaw storage at $4^{o}C$. During this period the blood had acceptable levels of extracellular free haemoglobin and potassium whilst the intracellular potassium and organic phosphate content was still satisfactory after five days.

In vivo survival studies were performed in healthy volunteers using radioactive chromium (^{51}Cr) as a red cell label and radioactive ^{125}I iodinated human serum albumin to measure the plasma volume. Complete units of frozen-washed red cells were transfused to the original donors and the survival of the lagged cells was measured at 24 hours and at weekly intervals after that for four successive weeks. The results obtained for five-day old (post-thaw) blood were compared to those of previously frozen-washed erythrocytes infused immediately

after processing. Both the 24-hour survivals and the half-life times were comparable in either case.

We have also investigated the leucocyte and platelet content of blood frozen and processed according to our protocol, by several different methods. Using the conventional haematological techniques of counting we found that the final red cell suspension contained 5-6% of the original leucocytes in the form of intact lymphocytes, whilst the polymorphnuclear cells and platelets disappeared completely. The viability of these cells was assessed by two different methods. The dye exclusion technique showed the absence of any viable cells whilst a low response to PHA stimulation was detected in lymphocyte culture.

However, using the radioactive assay technique in which the cell membrane of either lymphocytes, granulocytes or platelets was labelled with ^{125}I - Na - I and added to the blood before freezing, we found that approximately one third of the labelled leucocyte material and 2% of the platelet material remained in the final product after processing. However, most of the remaining leucocyte-derived material could be removed either by passing the blood through a Swank Blood Transfusion Filter or by removing the buffy coat after each centrifugal wash step. Although the latter technique was more efficient, it results in higher loss of red blood cells. When the remaining leucocytes were cultured in vitro with incompatible lymphocytes in a one-way mixed lymphocyte reaction no evidence of transformation could be detected indicating that these cells were not antigenic to foreign cells. However, when the antigen status of the frozen blood as a whole was studied in vivo by injection into laboratory animals and

the results were compared to those of fresh blood, we found that frozen-washed blood was able to stimulate antibody production against human lymphocytes. However, the levels of antibody produced from the injection of fresh blood were much higher than those induced by the infusion of frozen-washed red cells, whilst the latter could be diminished further by passing the blood through a Swank Blood Transfusion Filter prior to injection.

Following these experimental studies a frozen blood bank was established for therapeutic use. Since the establishment of the bank, frozen blood is given to the following categories of patients:

1. Patients suffering from thalassaemia major.
2. Patients in the medical renal dialysis and transplant unit.
3. Chronic anaemic patients who require regular blood transfusions.

Experiences with more than one thousand unit transfused to these types of patients showed the efficacy of frozen blood in restoring their haemaglobin levels to normal and in preventing Jebrile transfusion reactions in multiple transfused patients, which would otherwise occur with fresh blood transfusions.

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INTRODUCTION

HISTORY

The evolution of blood transfusion has been reviewed by Zimmerman in 1942 and by Hurn in 1968. Blood has always been linked with life and health. One of the earliest beliefs was that blood carried the secret of life which flowed out of the body with the streaming of blood. For this reason blood has always been used as a drug for restoring life and improving health. Ancient Egyptians have used baths of blood for resuscitation and treatment (Zimmerman 1942). Roman spectators used to rush into the arenas to suck the flowing blood from the necks of the dying gladiators.

The consideration of blood for transfusion only started after the discovery of the circulation by Harvey in 1616. This discovery stimulated interest in infusion and transfusion. Thus the first intravenous infusion was performed forty years after Harvey's discovery by an English astronomer and architect Christopher Wren. Using a bladder tied to a quill, Wren was able to infuse some drugs into the veins of dogs (Zimmerman and Howell 1932).

The first blood transfusion from one dog to another was also performed in England by the British anatomist and physician Richard Lower in 1665. One dog was bled to the point of death, then, its vein was connected to the artery of another healthy dog. The operation was highly successful and both animals survived.

Jean Baptiste Denis of France in 1667 carried out the first human transfusion using sheep's blood. Denis's first trial seemed successful, but repitition of the process into the same patient ended with the first mortality from blood transfusion. A complaint from the widow

against Denis brought about a parliamentary act to ban the whole operation. Following this disaster the first chapter in the history of blood transfusion was closed.

A hundred and fifty years passed before James Blundell, an Egnlish obstetrician, revived the interest in blood transfusion once more. Inspired by the high mortality rate among women suffering from post-partum haemorrhage, Blundell raised the question of restoring the lost blood to these patients. Using a more scientific approach than his predecessors, he began experiments on animals and came to the conclusion that only blood from the same species was effective in saving animals suffering from fatal haemorrhage.

Based on this theory Blundell performed the first human transfusion with human blood. All the first four trials failed, but because he was convinced and determined to succeed, Blundell carried out several other experiments which were all successful. Blundell also introduced a three-way syringe for transferring the blood from the donor to recipient without undergoing vascular anastomosis. This success brought back blood transfusion practice into notice and led to its spread to many countries. Also the indications for blood transfusion were extended from cases of anaemia and acute haemorrhage to include chronic infections and debilitating diseases, burns, uremia, syphilitic and mad patients. However the operation remained dangerous and surrounded with some mystery; success in one case followed failure in another.

In the year 1900 the problem was eventually solved by the discovery of the human blood groups and iso-agglutinins by Landsteiner. Soon after this discovery immunological tests for grouping and selecting

donors were introduced and thus the hazards of blood transfusion were eliminated and widespread application was possible.

In spite of the adoption of blood transfusion in many countries, the actual technique of the operation remained difficult, tedious and complicated. Further more, emergency operations were practically impossible. At first blood had to be transfused by direct anastomosis of the donor's artery with the recipient's vein, an operation which was full of potential complications. Later, many devices were introduced for direct transfusion of fresh blood; however, clotting within the apparatus constituted the major difficulty in application. It was, therefore, evident that in order to make maximum use of blood transfusion in practice, methods of preserving the blood unclotted must be found.

In 1914 Hustin in Belgium discovered the effect of sodium citrate in preventing coagulation of blood before transfusion. This was considered a major achievement in the practice of blood transfusion because for the first time it became possible to separate the recipient away from the donor during transfusion. Hustin made no attempt to preserve the blood which was used immediately after collection. Nevertheless, later experience with citrated blood showed that it had a tendency for rapid haemalysis if stored for more than one week.

In 1916 Rous and Turner discovered that the addition of glucose to the blood-citrate mixture greatly prolonged the shelf life of the blood without loss of its viability. The preservative mixture suggested by them was as follows:-

2 parts of isotonic citrate (3.8% Sodium citrate in water)

5 parts of isotonic dextrose (5.4% dextrose in water)

3 parts of blood

Rous-Turner mixture was efficiently used during World War I and remained unchallenged after that for almost two decades. However the beneficial effect of glucose in delaying haemolysis has not been explained until twenty-five years later, when Aylward et al (1940) and Maizels (1941) suggested that it is metabolized by the red cell. In this way the breakdown of organic phosphates, which otherwise took place, was delayed. Glucose also helps the red-cell membrane to maintain its relative impermeability to cations and thus prevents swelling of the red-cell on storage (Maizels 1941). Furthermore, the progressive increase in red-cell fragility which normally occurs on storage in the absence of glucose is greatly reduced in its presence (Maizels 1941 - Rapoport 1947).

"Just as one cannot draw money from a bank unless one has deposited some, so the blood preservation department cannot supply blood unless as much comes in as goes out". On this simple principle Fantus (1937) started the first "blood bank" at the Cook County Hospital in the United States. The establishment of this "blood bank" has made the use of stored blood an easy technique in America and Europe. Storage of blood had greatly economized both time and effort and made available more blood for transfusion.

By the year 1940 the anti-coagulant-preservative mixture used in the United Kingdom was the M.R.C. solution and was composed of:-

100 ml 3% trisodium citrate

20 ml 15% glucose

to be mixed with 420 ml of blood

However because the citrate solution has an alkaline reaction, caramalization of glucose was inevitable when the two compounds were

autoclaved together. Each solution was autoclaved separately and then both are mixed under aseptic conditions (Loutit et al. 1943).

Acidification of the preservative mixture was originally suggested by Maizels and Whittaker in 1940 when they found that it had a beneficial effect in preventing in vitro haemolysis during storage.

Loutit et al. 1943 investigated the effect of acidification of the glucose-citrate mixture on prevention of caramalization of glucose during autoclaving. They reported that acidification not only inhibited caramalization but also improved the preservative power of the mixture as evidenced by in vivo survival of the red cells after infusion. Loutit and Mollison 1943 recommended the following solution to replace the standard M.R.C. mixture:-

Disod. citrate	(monohydric)	2g
Dextrose	(anhydrous)	3g
Water		120ml

to be mixed with 420 ml of blood.

Further investigations showed that a decrease in the pH of the preservative solution, adds to the effect of glucose in slowing the progressive increase in fragility during storage and in retarding the permeation of cations.

By the year 1947 it was obvious that whole blood could be collected into ACD anticoagulant, stored at +4°C for 21 days and still remain clinically acceptable for transfusion. However at the end of this period the 24 hour post-transfusion survival is only 70% (Valeri 1968a), the oxygen transport function has deteriorated (Valtis and Kennedy 1954), and the 2,3 DPG content has reached zero (Blagdon 1972). Because of the progressive deterioration of the blood in ACD much effort has been

devoted to prolonging the storage period of blood as well as to improve its physiological function after storage.

Gibson et al. (1957) suggested that the viability of stored blood collected on ACD anticoagulant was mainly determined by the amount of damage imposed during collection, "lesion of collection". In order to minimize the severity of this lesion they developed a "Citrate-phosphate-dextrose" (CPD) preservative mixture. The use of such preservative has extended the shelf-life of stored blood from 21 days to 28 days. Compared with ACD, CPD showed slightly less haemolysis, decrease in the amount of potassium leak from the cells and higher in vivo survival. Furthermore the citrate content was 20% less than ACD, and this means decreased risk of citrate intoxication and serum calcium depression. Finally, CPD was found to be a better medium for the maintainance of the red-cell 2,3 DPG (Mollison 1972).

Supplementation of either ACD or CPD with purine nucleoside, adenine, maintained the red-cell viability for a longer period than CPD alone (Mollison 1972). Akerblom et al (1967) reported their experience with over 5,000 transfusions of blood collected on ACD - adenine. No untowards clinical reactions were observed with the use of this blood even after multiple transfusions. They recommended its use without reservation for transfusing adult recipients, however, care should be taken with exchange transfusion in newborn infants.

FREEZING

Adequate supplies for all contingencies has long been the ambition of blood banks throughout the world. It is important to insure availability of blood in time of major disaster and to compensate for the fluctuation of supply and demand. Stored blood progressively loses its viability. This loss of viability is believed to be due to continuation of glycolysis outside the body (Sloviter and Ravdin 1965). Slowing of the rate of glycolysis and thus improvement of viability could be achieved through reduction of the storage temperature. Thus storing of blood at $+4^{\circ}C$ prolonged its shelf-life to three weeks. It is regretable that additional prolongation of the storage period through further decrease of temperature is impossible, for the extent of the reduction of temperature is limited by the fact that red blood cells tend to haemolyse rapidly if frozen below $-3^{\circ}C$ (Meryman 1964). A solution for this problem was found by Polge, Smith and Parkes (1949) when they discovered that glycerol could protect fowl spermatozoa against freezing injury. Luyet in 1949 also reported his success in recovering intact red blood cells after very rapid freezing and thawing, $200^{\circ}C$ per second, without any protective. Thin layers of blood supported on glass slides were rapidly frozen, by direct immersion in liquid nitrogen, then rapidly thawed in warm saline. Most of the cells were recovered morphologically intact.

Smith, in 1950 extended and adapted the work of Polge et al. to the red blood cells. She showed that, in the presence of glycerol, human red blood cells could be frozen and thawed without major haemolysis.

Following these findings preservation of blood by freezing has followed two different lines. One method utilized rapid rates of cooling with extracellular additives and the other slow cooling rates in the presence of glycerol or similar compounds that penetrate the red cell membrane.

In order to produce its action, glycerol has to permeate the red cell (Lovelock 1953b). Ironically it is this permeation that has produced many of the practical problems, because glycerol has to be washed out of the cell before transfusion (Sloviter 1951a). Furthermore, high concentrations of glycerol, (4.0 M) are required, to produce full protection, and the removal of such a concentration of this viscous material has presented major difficulties.

Rapid freezing in the absence of additives, as proposed by Luyet (1949), necessitates the use of very high cooling rates, 200°C per second, and difficulties in obtaining such high rates with large volumes of blood prevented its application in practice. Addition of sugars and polymers, as extracellular cryoprotective agents, has made it possible to freeze large quantities at less rapid cooling rates and at the same time maintained high recovery of red cells (Meryman and Kafig 1955, Doebbler and Rinfret 1962). Obviously the use of extracellular compounds does not call for post-thaw washing, the so called "one step approach". However, none of the extracellular cryoprotectants have been shown to give clinically acceptable products without post-thaw manipulation (Meryman 1968b).

It was such difficulties that led to the development of a method which utilized a mixture of low concentrations of sugars and glycerol (relatively easy to remove) coupled with intermediate rate of freezing (Pert et al 1963, Krijnen et al, 1964).

GENERAL AND PHYSICAL ASPECTS OF FREEZING

Ice crystal formation does not normally begin as soon as the freezing point of a solution is reached. A nucleus must be present in the solution for ice to condense around it. This nucleus is not formed of ice, except at very low temperatures, but of some other particle simulating ice in configuration and happening to be present by chance in the liquid phase. The size of the nucleus necessary to initiate ice crystal growth depends upon the temperature of the solution cooled (Meryman 1963). The higher the temperature the larger the size needed. Around the true freezing point the size of the crystal nucleus is quite large and the chance of finding such a large sized nucleus is very low. Thus as the temperature drops below the true freezing point no freezing takes place and the solution is said to be supercooled. With further drop of temperature the required size of the nucleus necessary to start crystallization becomes less stringent and eventually an existing nucleus becomes of critical size and thus water begins to condense around it.

The change of phase from liquid to solid greatly limits the movement of the water molecules and thus reduces the consumption of energy. The excess energy is released as heat "latent heat of fusion" and unless this heat is rapidly removed from the growing crystals, the temperature of the solution will rise to the true freezing point and remain there until the transition phase is complete.

In almost every living cell or tissue, slow freezing results in extracellular nucleation and ice crystallization. An exception to that are tissues which have been killed by previous freezing and

thawing, further freezing of these tissues results in ice crystal formation throughout the media (Meryman 1956 - Hurn 1968). Again when the rate of heat removal from the medium is slow, the few growing ice crystals will be sufficient to balance all the heat removed by the coolant and the temperature of the solution will remain at the freezing point where further nuclei will be of the critical size.

As the ice crystals formed grow they extract water from the surrounding fluid and thus the electrolyte concentration of the extracellular fluid rises. The resulting high osmotic pressure removes more water from the cells which thus become shrunken. This process proceeds until all the available free water is consumed and the cells are left suspended in a highly concentrated solution.

On the other hand, if the heat resulting from ice crystal growth is removed rapidly, the few crystals formed will not be sufficient to balance the heat lost to the coolant. Under these conditions the temperature of the solution will continue to drop and thus more nuclei become of critical size throughout the field and water crystallizes around them until eventually the number of ice crystals become sufficient to balance the heat removed. For this reason it is said that rapid freezing results in the formation of numerous small ice crystals which are uniformly distributed throughout the specimen but predominantly intracellular (Meryman 1956, and Meryman 1963).

There is a considerable confusion in the literature as regards the meaning of cooling rates. Cooling rates are often classified into slow, rapid and ultrarapid. However, all these mean different things to different investigators. Thus Luyet et al (1963) described a cooling rate ranging from 25°C to 250°C per second as rapid for blood

frozen in capillary tubes. Rinfret (1960) sprayed water on the surface of liquid nitrogen and reported a rapid rate about 50°C per second. Doebbler and Rinfret (1962) using aluminium tubes of elliptical section for freezing 5 ml quantities of blood reported an average cooling rate about 10°C per second and the rate was described as rapid. Strumia et al (1958a, 1960a) reported a cooling rate from 3 to 7°C per second when a very thin flat metal container or Teflon bag was cooled in dry ice-ethanol bath.

Pert et al (1964, 1965) and Krijnen et al (1965) used a rapid rate of cooling to freeze blood suspended in low concentration of glycerol. The average cooling rates described by Pert et al. (1963) ranged from 0.36 up to 6.7°C per second when they immersed Teflon bags containing 100 ml of glycerolized blood in liquid nitrogen and changed the thickness of the surrounding layer.

This shows that the term "rapid" as used by the five groups of investigators was very imprecise as it reflected a wide range of velocities without consideration of the volume of sample under investigation or the presence of protective agent. Luyet and Rapatz (1970) classified the rate of cooling into three categories:-

(1) Rapid cooling rate: from 100 to 25°C per second

(2) Medium rate: from 5 to 1°C per second

(3) Slow rate: from 5 to 1°C per minute

They considered a rate higher than 100°C per second or slower than 1.0°C per minute as very high and very slow respectively.

Hurns (1968) described cooling rates in various approximations, thus 1°C per minute was described as slow cooling, a 100°C to 1000°C per minute as rapid cooling and a rate higher than 10,000°C per minute

as ultrarapid.

Meryman (1956) suggested basing the concept of slow or rapid freezing on the location of ice crystals. Thus the term "slow freezing" designates conditions under which only extracellular crystallization develops. "Rapid freezing" would indicate the conditions in which the cooling rates are sufficiently high to produce intracellular crystal formation.

SLOW FREEZING

This type of freezing could be achieved by placing the material in a conventional deep freeze. Ice crystals are formed by the so called "heterogeneous nucleation" (Meryman 1956 - Hurn 1968). As has been mentioned the crystals of ice are few in number and grow around a nucleus simulating ice that is present by chance in the liquid phase. The slow rate of freezing leads to the formation of ice crystals only outside the cell. As the crystals of ice grow the osmolarity of the extracellular fluid increases and water is removed from within the cell and added to the growing crystals the cell itself becomes crenated. This process of water withdrawal and ice crystal growth continues until all the "available freezable" water is consumed and the cells are left in a concentrated solution (Meryman 1956, 1963). Many types of cells, epidermal and muscle cells, can withstand freezing in this way without suffering appreciable damage, for on rewarming the cellular water is reimbibed and the cell resumes its histological features and physiological functions (Meryman 1956). Red cells however are notable exception, they are immediately hemolyzed after slow freezing once

the temperature drops below -4°C (Lovelock 1953a).

Damage from this type of freezing is primarily attributed to the increased concentration of electrolytes, particularly sodium chloride, which results from freezing of water out of the solution (Lovelock 1953a, Meryman 1956). High concentrations of glycerol may be used to protect against this type of injury. Glycerol, as well as other similar compounds, produces its action by reducing the amount of ice formed. These compounds tie up a great proportion of water making it unavailable for crystallization while it is still available as solvent for the electrolytes. In doing so the concentration of electrolytes and cell solutes are thus reduced and haemolysis may be minimized (Lovelock 1953a). Meryman (1956) maintained that one molecule of glycerol can bind up three moles of water and prevent it from freezing. Glycerol, in order to be effective as a protective agent, has to penetrate the red cell (Lovelock 1953b) and it has been shown that those cells which are rendered impermeable to glycerol, e.g. in the presence of copper ions, are not protected by it. However, it is the permeation of glycerol into the red cell which constitutes all the difficulties surrounding its use. Because of its viscosity, removal of high concentration of glycerol presents a major drawback for this method (Meryman 1964). The principal obstacle was that of the washing procedure. Continuous-flow centrifugal washing was completely ineffective in the early days and as a consequence wash solutions of varying composition and hypertonicity were required to minimize osmotic haemolysis. Excessive hypertonicity of these solutions, particularly when they were non-electrolytes, led to electrolyte loss from the cells (Meryman 1968b).

MECHANISM OF FREEZING INJURY

1. Salt concentration theory:

The ability of investigators to preserve red blood cells for prolonged periods at subzero temperatures has turned their attention away from investigating into the mechanism of freeze-thaw injury and the mode of additive protection. Thus most of the efforts have been devoted to the empirical development of methods of freezing. Fortunately, however, the subject of freeze-thaw injury and the mechanism of its prevention has attracted the interest of a few investigators. One of the pioneers in this field was J.E. Lovelock who, in 1953, suggested the salt concentration as a cause of freezing damage.

At first the observation that slow freezing leads to the formation of large plaques of ice crystals that might compress the red cells between them has led to the assumption that mechanical pressure of ice was the principal cause of freezing damage (Luyet 1949, Smith et al. 1951, Glauser and Talbot 1956). However, the demonstration that red cells could be frozen solid to temperature lower than $-3^{\circ}C$ without appreciable haemolysis (Strumia 1949) and that intact animal extremeties can survive freezing for short period (Meryman 1963) has proved that the physical presence of extracellular ice crystals alone is not necessarily lethal. As an alternative to the straight forward mechanical injury, it has been proposed that the injury is a biochemical one resulting from the increased concentration of electrolytes that develop from the separation of water into ice (Lovelock 1953a). Working with human erythrocytes Lovelock found that slow freezing to $-4^{\circ}C$ and very rapid freezing to $-79^{\circ}C$ both gave equally good recoveries of red cells. This led to the conclusion that lysis occurred not because of the presence of solid ice, but because of some other factor

that becomes active on slow freezing to temperature lower than $-3^{\circ}C$. Lovelock also demonstrated that the temperature range in which maximum haemolysis takes place lies between $-3^{\circ}C$ and $-40^{\circ}C$ for cells unprotected by cryoprotective agents. He estimated that this range must be passed through in less than three seconds if damage is to be avoided. Further experiments showed that the upper limit of this critical region is identical with the freezing point of 0.8M sodium chloride solution while the lower limit corresponds to the eutectic point of the intracellular salt content. This was taken by Lovelock as an indication that the critical region of temperature was that range during which the red cells are exposed to salt concentration higher than 0.8M. Accordingly he suggested that the freezing damage was caused by increased concentrations of electrolytes within and without the cell due to the removal of water in the form of ice. Thus haemolysis starts at $-3^{\circ}C$ when the amount of water removed is sufficient to raise the salt concentration in the fluid phase to 0.8M. A further drop in temperature results in solidification of more and more water and increase in salt concentration until at $-40^{\circ}C$ when all the water within and outside the cell has frozen solid and the salts are no longer available to exert their damaging effect. If the electrolyte concentration theory is to be accepted, then it must be expected that suspension of red cells in fluid medium containing increasing salt concentration must give the same haemolysis caused by freezing; Lovelock was able to demonstrate this. Furthermore, he has been able to show that even in the presence of glycerol, haemolysis of red cells always starts once the concentration of sodium chloride reaches a mole fraction of 0.014 (0.8M) regardless of the concentration of glycerol or the temperature reached (Lovelock 1953b).

Lovelock's assumption was confirmed later by the work of Farrant (1965) who showed that living organised tissue frozen in a system specially designed to prevent rising electrolyte concentration during cooling survived and regained its normal function after rewarming in spite of the development of ice crystals.

As a mechanism of injury Lovelock (1953a) suggested that:-

1. Exposure of the red cells to salt concentration higher than 0.8M renders them permeable to sodium ions. Haemolysis then takes place either slowly in the suspending medium or rapidly when the erythrocytes are restored to isotonic media. Freezing and thawing probably carries the cells through a similar change.

2. High salt concentration increases the sensitivity of the red cells to thermal shock and mechanical stress resulting from the formation of ice crystals.

3. Exposure of the red cells to sodium chloride concentration higher than 3.0M brings about the lyotropic action of sodium chloride and the cells are completely destroyed and loses its integrity.

It was therefore concluded that one aspect of the protective action of glycerol at low temperature is that it acts as a salt buffer.

Meryman (1956) discussed general principles of freezing and freezing injury in living cells. In his discussion, he supported the salt concentration theory of Lovelock and stated that there was no evidence that extracellular ice formation produced mechanical damage. When freezing is slow and ice crystals are extracellular, flexible cells can collapse passively during dehydration without suffering mechanical injury. If mechanical injury is not a factor, then cell

death results from the denaturing effect of high salt and solute concentration associated with the dehydration process. Since the resultant denaturation is a biochemical reaction it would proceed at both time and temperature - dependant rates. This would explain the cessation of injury at low temperature as well as the survival of the red cells after very rapid freezing and thawings.

The fact that damage suffered by the red cells is arrested once the temperature is below a certain point is of great practical implication. This phenomenon has been attributed to the eutectic of the cell contents (Lovelock 1953a). Thus, when all the intracellular solvent have frozen solid the salts are no longer available to produce its injurious effect. However subsequent experiments with red cells suspended in glycerol containing media showed that while glycerol can lower the temperature at which haemolysis starts, it does not shift the lower limits of the critical region. This implies that, in the presence of glycerol, haemolysis still continues to decrease at $-40^{\circ}C$ while the phase transition is still incomplete. This also means that while glycerol can delay the rise in salt concentration at the early stages of the critical region, high concentrations might still be expected in the lower temperature ranges. This suggests that the external sodium chloride concentration is not the sole factor in determining cellular damage. Attention has thus been directed to the intracellular salt concentration particularly potassium chloride, and Lovelock (1953b) proposed that unlike sodium chloride, the solubility of potassium chloride progressively decrease with the fall of temperature and below $-40^{\circ}C$ potassium chloride becomes insufficiently soluble to maintain the lethal concentration of 0.8M.

2. Mechanical Injury:-

Luyet in 1949 originally attributed the damage from which the red cells suffer during slow freezing to the mechanical deformation of ice crystals within the cells and explained the benificial effect of ultrarapid freezing on the basis of prevention of crystal formation. However what was described by Luyet in 1949 as slow freezing (few degrees per second) is no longer valid in the context of blood preservation by the present methods. Furthermore it has been realized that slow freezing leads only to formation of extracellular ice crystals. Nevertheless Glauser and Talbot (1956) considered that even the presence of extracellular ice may be sufficient to cause part of the haemolysis observed on freezing and thawing. Their hypothesis was based on the observation that additives which prevent haemolysis are strong hydrogen bonders and thus cause distortion of the ice lattice. Also, high molecular weight hydrogen bonding additives protect against haemolysis although they do not enter the red-blood cells. They thus, attributed the damaging effect of extracellular ice to the migratory recrystallization which takes place in the frozen state but becomes progressively less and less at temperature below $-60^{\circ}C$. Lusena and Rose (1956), using hog's blood, studied the effect of rate of ice crystal growth on haemolysis during freezing and thawing. They observed that the amount of haemolysis increased sharply when the rate of ice-crystal growth reached 0.7 cm per second in the absence of glycerol and 0.3 cm per second in its presence. They discussed the possible mechanism that made a slow rate of ice crystal growth desirable and attributed, in part, the protection afforded by glycerol to its retarding effect on crystal growth.

Nei (1967, 1968a-1968b) has also investigated the mechanism of haemolysis during freezing and thawing at temperatures between zero and $-10^{\circ}C$ and studied the relationship between formation of ice and packing of the cells in liquid channels at decreasing temperature. They observed that within certain limits, increases in the concentration of cells in saline suspension resulted in increase of haemolysis after freezing and thawing. Sedimentation of erythrocytes before freezing or addition of other cells also increased the amount of haemolysis following freezing. An increase of salt concentration brought about a reduction of haemolysis. Packing of cells suspended in concentrated sodium chloride caused severe haemolysis even without freezing. Therefore, it was concluded by the author that the increased salt concentration is not the principal cause of haemolysis and that mechanical stress resulting from cell concentration is one of the most important factors of injury in freezing at near zero temperature.

3. Membrane damage:-

Although the salt concentration theory of freeze-thaw injury is the most widely accepted theory, the mechanism by which increased salt concentration might damage the cell is less clear. The fact that the cell membrane acts as a limiting barrier between the cell and its surrounding, exposes it to the brunt of the first assault brought about by the physical and chemical changes in the surrounding medium, as a result of freezing and thawing. Realization of this has led many investigators to concentrate on the physical, chemical and biological properties of the cell membrane to explore the mechanism and nature of freezing injury.

It is generally agreed that freezing damage is due to abnormal permeability of the cell membrane to nondiffusable solutes or cations, but the way this abnormality develops is a matter of controversy. Thus Lovelock (1957) explained the red cell injury on basis of denaturation of lipid protein complex of the cell membrane. According to him lipid-protein complexes are important constituents of the cell membrane of all living cells including the red cell. These complexes are held together and to the rest of the membrane by labile bonds which cannot stand vigorous changes in the surrounding environment. Lovelock suggested that the increase in the ionic strength of the suspension medium incurred by freezing is sufficiently great to bring about the destruction of the lipid-protein complexes. He showed that the level of phospholipids in the suspension medium steadily increased as a function of the concentration of sodium chloride in the solution and presumed that the loss of these phospholipids renders the cell membrane permeable to the cation sodium. As a result of this the red cells gradually swell and haemolyse when transferred back to isotonic medium as occurs in thawing.

Dehydration of the surface of the red cell was also suggested as a mechanism of freezing injury (Doebbler and Rinfret 1962). Those authors proposed that, as freezing proceeds, the red cells are subjected to an environment of continuously decreasing amount of "free" water and increasing concentration of salts. In their hypothesis a portion of the water of the red cell differ from "free" water and may be positioned at the cell surface. Disruption of the hydration structure of the red cell membrane could be a possible mechanism of freezing injury together with the chemical action of salt or the mechanical

stress of ice crystals. A confirmatory support of this view came from the work of Valeri et al (1966) who studied the relationship between the in vivo survival of previously frozen red cells and their physical structure and metabolic state. They concluded that freeze-thaw injury might be a membrane lesion and that cryoprotective additives protect the red cells through preservation of its surface hydration. However, Meryman (1968a) found no relation between freezing injury and any specific action of electrolytes. On the other hand he observed that this injury usually resulted from excessive concentrations of any non-penetrating extracellular solute which created an osmotic pressure gradient across the cell membrane. This pressure gradient led to leakage of solute into the cell rendering it osmotically hypertonic, sudden transfer of these cells back to a physiological medium results in their haemolysis. In the absence of such a gradient, very high solute concentration, including electrolyte solutes, were unable to produce haemolysis and Meryman was able to demonstrate that ammonium acetate, a penetrating electrolyte, in high concentration is equivalent to glycerol in protecting against freezing injury.

Further confirmation of Meryman's minimum cell volume hypothesis came from the work of Farrant (1972) and Farrant and Woolgar (1972a and 1972b) who showed that exposure of the red-cells to the effect of hypertonic solution of sodium chloride or sucrose led to crenation of the red-cell volume until it reached a minimum at 2000 m. Osm./Kg water. Beyond this limit the cell resisted any further shrinkage, and leakage of cations to and from the cell took place. However, although there was evidence to indicate that a net uptake of solute occurred in cells exposed to hypertonic solutions of sodium chloride,

as observed by Meryman (1968a), Farrant and Woolgar 1972a and 1972b found no evidence for a net uptake of solutes in those cells exposed to hypertonic solutions of sucrose. They, therefore, suggested that post hypertonic haemolysis was probably of colloid osomotic nature. This means that when the cell is permeable to cations, the osmotic pressure due to intracellular haemoglobin, (if unopposed by extracellular impermeant solute) would cause the cell to swell and eventually rupture, when transferred back to isotonic media. If the cells were suspended in a hypertonic penetrating additive like dimethylsulfoxide (DMSO), the latter will rapidly permeate the cell and hence prevents it from shrinking. Under these conditions the osmolality of 2000 m. Osm.would be passed without leakage taking place. This was taken by Farrant (1972) as an indication that cation leakage is mainly determined by cell shrinkage and that penetrating additives protect against freezing injury by delaying the sodium leak to a higher osmolality, in other words it lowers the freezing temperature.

Sumida and Toshihiko (1968) also studied the membrane permeability of fresh, frozen and liquid stored ACD blood. They measured the intracellular potassium and sodium and using an arbitary formula worked out the resting membrane potentials before freezing and after thawing. They observed that the potentials were significantly lower in frozen and ACD blood in comparison with the fresh blood. They, thus, concluded that the membrane permeability of the ACD and frozen cells was dependant on the passive rather than on the active transport of the membrane and would be easily affected by the fluid change outside the cell with endosmosis taking place in these cells with abnormally lowered potentials. However, the change in permeability above 1800 m. Osm/Kg water found by Farrant and Woolgar (1972a)

occurred at membrane potentials of 22.4 mV (inside positive) or at 19.0 mV (inside negative) depending on the composition of the extracellular solution. This was taken by Farrant and Woolgar (1972a) and Woolgar (1974) as evidence that the change in permeability was not dependent on a specific membrane potential.

Grieff and Seifert (1968) postulated that the mucopolysaccharides on the external surface of the cell membrane are cryosensitive sites. Because they are closely connected with other biochemical units of the cell membrane, physical changes in these sites, may affect the amount of injury to transport mechanisms for the movement of ions and proteins across these highly selective, semipermeable barriers. They developed a membrane adsorption test for determining the effect, on those cryosensitive sites, of:-

1. different rates of cooling (slow, intermediate and rapid)
2. Cyclic freezing and thawing
3. storage in the frozen state at low temperature
4. the addition of various types of additives.

They observed that the amount of damage to the cryosensitive sites was inversly proportional to the rate of cooling. However once frozen, storage did not add to the initial damage, but the latter was progressively increased by cyclic freezing and thawing. Minimal damage occurred in the presence of DMSO or glycerol. They thus concluded that protective agents act by external binding with the sensitive sites of the cell membrane.

MODE OF ACTION OF GLYCEROL

The cryoprotective property of glycerol was discovered in 1949 by Polge et al., when they found that fowl spermatozoa could be frozen and thawed, without losing motility if suspended in a medium containing glycerol. Smith (1950) was able to adapt the protective action of glycerol to the red cells. Glycerol has since been the favourite cryoprotective additive and in fact all the methods of cryopreservation of blood in current use today depend on its ability to prevent red cell haemolysis which would otherwise occur on freezing. However, despite this wide acceptance, the mechanism by which glycerol produces its action has remained unclear, though several theories have been advanced.

One of the earliest investigations into the mechanisms of protection afforded by glycerol was carried out by Smith et al. (1951) who observed directly under the microscope the consequences of addition of glycerol to red-cell suspensions. They reported that glycerol modified the pattern of ice from coarse broad-plaques, which were compressing the red-cells, into delicate feathery-like crystals. In addition, a substantial amount of fluid was left unfrozen forming narrow channels in which the erythrocyte were seen lying unaffected. Although the authors did not attribute the mode of action of glycerol, particularly, to these alterations, they suggested that the mechanical stress of freezing must have been greatly reduced by its presence.

No doubt, glycerol substantially minimizes the mechanical effect of ice crystals in the erythrocytes, but to ascribe its action completely to this effect is hard to believe. A more sensible theory was advanced by Lovelock (1953b), who suggested that glycerol acts as

a salt buffer. He demonstrated that the concentration of electrolytes, which increases when a solution of sodium chloride is frozen, decreases with increasing the glycerol concentration until in the presence of 2.5 M glycerol the lethal salt concentration, 0.8 M, is not reached in the temperature range 0 to -40°C.

For the protective action to be fully expressed, glycerol has to be present both intra - and extra-cellularly. If it is prevented from entering the red cells, by the addition of copper ions, they are no longer protected against freezing injury (Lovelock 1953b). This observation has led to the assumption that intracellular salt concentration is, at least, as important as the extra cellular sodium chloride in producing freezing damage (Lovelock 1953b).

The effect of glycerol in buffering the increased salt concentration was totally explained on the basis of its colligative property (Lovelock 1954). This means that the more the mole fraction of the solute is increased, the less the amount of water is frozen, the greater the reduction in the electrolyte concentration. Thus it is possible to maintain the salt concentration below the lethal level at any given temperature simply by adding a sufficient amount of the additive.

The protective action of glycerol and all cryoprotective additives has been related not to the molarity of the solute, but to the number of potential hydrogen-bonding groups provided by the additive (Doebbler and Rinfret 1962). Those authors suggested that H-bonding protectives, including glycerol, serve to stabilize the surface hydration of the cell. This would mean that the protection afforded by glycerol, and other solutes is the result of specific rather than simple colligative property.

In 1968a Meryman examined the effect of increased salt concentration on the volume of red cells. He found that as the salt concentration of the surrounding increases, cell volume gradually decreases by losing more water to the external environment. This goes on until a certain volume is reached below which the cell cannot shrink any more. Haemolysis of the red cells is thus the result of membrane damage produced by the development of an "osmotic pressure gradient" across the cell membrane greater than the cell can compensate for by reducing its volume. The argument that freezing damage is the result of denaturation of the cell membrane by the strong sodium chloride concentration is off-set by the demonstration that identical results have been produced in the presence of concentrated solution of sorbitol or sucrose (Meryman 1971b). According to this model the role of the cryoprotective additive is to minimize the increase in the extracellular salt concentration, absolutely by a colligative action (Meryman 1968a-1971a and 1971b, and Meryman and Hornblower 1972b).

Meryman further assumed that any solute which equilibrate rapidly across the cell membrane, whether it is an electrolyte or non-electrolyte, would reduce the salt concentration and if present in sufficient amount would prevent completely the shrinkage of the cells beyond their tolerable limits (Meryman 1971b). However for any solute to exert this effect, it is essential to be freely penetrating to the cell membrane otherwise it will add to the osmotic pressure of the extracellular environment by itself (Meryman 1968a, 1971a, 1971b, and Meryman and Hornblower 1972b). Again the solute has to be non toxic in the highest concentration used and must be used at multimolor concentration as required according to the rate of freezing.

GLYCERAL CONCENTRATION AND STORAGE TEMPERATURE

Smith in 1950 discovered that small aliquots of human red blood cells could be frozen rapidly to -79°C and recovered intact if 10 to 20% glycerol was added to the blood. In 1951a Sloviter reported that equal volumes of whole blood and 30% (W/V) glycerol were frozen rapidly to -79°C in a test tube, held at this temperature for two minutes and then thawed, 79 to 94% of the cells were recovered intact. Experiments with rabbit blood (Sloviter 1951b) showed that, these frozen cells also had normal in vivo survival. However, when Sloviter's technique for freezing was attempted with large volumes of blood, e.g. with 500 ml volumes, the rapid rate of cooling could not be maintained and poor red cell recovery was obtained (Mollison et al. 1952).

Chaplin and Mollison in 1953 discovered that when the concentration of glycerol was increased to 30% W/V (final concentration) the in vitro red cell recovery became independent of the rate of cooling and thus freezing of pint volumes of blood was attained. However, storage of the blood in the frozen state at -79°C (Co_2 and alcohol mixture) proved to be fairly expensive and maintainance of that temperature at a constant level required frequent replenishment of the coolant. This led to the search for other methods of cooling and storage. Chaplin and Mollison (1953) attempted to store the blood at -20°C, and in order to prevent the excessive increase of the salt concentration intracellularly they substituted Trisodium citrate for the isotonic saline in the glycerol solution.

Thus the cryoprotective additive recommended by them consisted of:

Glycerol	400 ml.
Tri Sodium citrate	30 g.
Water to	1000 ml.

When equal volumes of this solution and packed red cells were mixed together they calculated that the final glycerol concentration of the fluid phase was approximately 30% W/V. After storage at -20°C for three months about 98% of the red cells were recovered intact.

Brown and Hardin (1953) have used a final concentration of glycerol about 40% w/v., but mixing such high concentrations of glycerol directly with the red cells brought about excessive haemolysis. For this reason the authors suggested adding the glycerol in two steps. First equal volumes of 30% W/V glycerol, in 0.16M sodium lactate, and erythrocytes are mixed and left to equilibrate at room temperature for some time. In the second stage, a solution of 50% glycerol (W/V) in 0.16M sodium lactate was added to give a final concentration of 40%. After storage of such blood at -15°C for 50 days, only 79% of the cells were recovered intact and on transfusion only 47% of them survived. By contrast storage at -70°C for 6¼ months resulted in 90% in vitro recovery and 64% post transfusion survival at 24 hours.

Chaplin et al. (1954) demonstrated that -20°C was an unsatisfactory temperature for long-term storage of human red blood protected by glycerol. They maintained that while red cells stored at -20°C have very little in vitro haemolysis, the decrease in post-transfusion survival was rather rapid. This rapid loss was acribed to the continuation of the chemical and metabolic process at this relatively high temperature and was demonstrated by the progressive diminition of the cellular glucose. Chaplin et al. (1957) compared the post

transfusion survival of chromium labelled red blood cells that have been stored at -20°C for 13 months with that which were preserved at -45°C for one and a half years. Both types of cells were frozen in the presence of buffered-citrate glycerol solution. They reported that while the cells that had been stored at -20°C showed a 24 hour post-transfusion survival of 58% those preserved at -45°C had a post-transfusion survival of 96% at 24 hours.

The idea of selecting -45°C as storage temperature was based on the assumption that the range of temperature in which maximum haemolysis occurs, lies between -3°C and -40°C (Lovelock 1953a). It was thus thought that -45°C was significantly below the critical temperature but still within the limits of refrigeration available at that time.

Although -45°C was a satisfactory temperature for storage of glycerolized red cells for up to 18 months, the results after six years storage were disappointing (Schmidt and Stohlman 1964). These authors reported a mean in vitro loss of 25.4% and a 24 hour post-transfusion survival ranged from 20% to 60%. These results suggested that -45°C was not cold enough to maintain the viability of the preserved red cells for very long duration, however -45°C was satisfactory for blood banks with a rapid turnover of frozen blood (= 6 weeks). Nevertheless this had put an end to further investigations at this temperature.

Previously Chaplin et al. (1956) demonstrated that red blood cells suspended in glycerol to a final concentration of 30% W/V and stored for 21 months at -79°C had in vitro recovery of 90% and in vivo survival of 80%. There was no evidence that the viability of the cells was influenced by the period of storage as these red cells which have been stored for 21 months behaved in the same way as those stored for only

one month. They also found that if the glycerol concentration of red cells stored at -79°C exceed 2.7M the electrolyte composition of the medium became relatively unimportant. Thus results obtained with glycerol-saline solution were as good as those obtained with glycerol-citrate solution.

A glycerol concentration as high as 7.0 M has also been tried for cryoprotection of the red cells but the results were discouraging (Jones et al 1957). Exposure of the cells to this very high concentration of glycerol, even for a very short period, resulted in inhibition of the glycolytic processes and diminished the post-transfusion survival. This confirmed the observation reported by Tullis et al in 1956 that the highest concentration of glycerol that can be tolerated by the red cells is 50%. At this concentration the haemolysis of the red cells resulting from freezing injury was minimal, but glycerol concentration higher than that were highly toxic. Tullis et al have also demonstrated that with 50% glycerol there was no need to use a rapid cooling rate and rigid control of the storage temperature was unnecessary.

In 1956 two important events in the history of the frozen blood occurred. The first was the adaptation (by Tullis et al , 1956) of the Cohn Blood Fractionator, a closed sterile semiautomatic continuous wash centrifuge, for both addition and removal of the glycerol from the red blood cells. The second was the construction by Harris Refrigeration Company of an electrical refrigerator with two stage and three stage compressor system to maintain storage temperature constant at -80°C and -120°C respectively. Following the introduction of these refrigerators a new programme for the preservation of red cells

in the frozen state was immediately developed by a group of investigators at the United States Naval Hospital in Chelsea, Massachusetts. This has, for the first time transferred the frozen blood from the research laboratories to the hospital wards for clinical use (Tullis et al 1958, Ketchel et al. 1958, Haynes et al. 1960, Haynes et al. 1962). Glycerolization was achieved in two steps 300 ml of 20%, then 1000 ml of 50% so that a final concentration of 50% W/V was obtained. The glycerolizing solution was made in isotonic-neutral sodium lactate (0.16 M) which also contained the following:-

0.2%	dextrose
0.004 M	potassium-chloride
0.004 M	magnesium chloride

Freezing was carried out slowly by placing the blood-glycerol mixture in the electrical refrigerator at either -80°C or -120°C where the blood was completely frozen and reached refrigerator temperature in eight hours. After storage for up to 44 months at the above temperature approximately 82% of the donor cells were recovered intact and on transfusion 90% of them survived at 24 hours. There were no apparent effects of the storage period on the in vivo survival of the preserved red cells.

Huggins in 1965 used glycerolizing solution made up of:

8.6 M	glycerol (79.2% W/V)
8.0%	glucose
1.0%	fructose
0.3%	disodium EDTA

The sugars were included to maintain the right osmotic pressure without adding to the ionic strength of the solution. The EDTA was added to

prevent the binding of complement to the red cells which would otherwise occur and results in a false positive Coomb's reaction. One volume of that solution was added to one volume of packed red cells, the mixture frozen slowly and stored at -85°C in a mechanical refrigerator for a maximum of one year and a half in the presence of EDTA (Valeri 1970) or two years without EDTA (Huggins and Grove-Rasmussen 1973).

After deglycerolization by the so-called reversible agglomeration in a cytoglomerator the in vitro recovery ranged from 70 - 82% with EDTA and from 80 - 85% without EDTA and the 24-hour post-transfusion survival, by the ^{51}Cr method, was approximately 70% in the presence of EDTA and about 80% in its absence (Valeri 1970).

Pert et al. 1963 introduced the low-glycerol intermediate-freezing rates in liquid nitrogen. They studied the relationship between cooling rates and the concentrations of the protective agent. They used five different cooling rates together with a concentration of glycerol which varied from 2% to 50% W/V. In terms of glycerol concentration the recovery curves obtained with the five cooling rates indicated that

1) At a glycerol concentration of 40% or more the recovery of the red cell was independent of the rate of cooling.

2) At glycerol concentrations of less than 14% rigid control of the cooling rate was essential for adequate recovery.

3) Glycerol concentrations between 20% and 40% required some control of freezing rate to avoid too fast a cooling rate.

4) A concentration of glycerol between 14% and 20% was the most ideal (Pert et al. 1965).

The glycerolizing solution suggested by these authors consisted of

14 - 16% W/V glycerol

2.76 g% sucrose

0.85 g% Na Cl.

The Teflon-bag containing the glycerolized blood was frozen by direct immersion in liquid nitrogen. After thawing and washing, 96 to 99.5% of the red cells were recovered intact and on transfusion 86 to 95% of them survived at 24 hours post-transfusion.

Krijnen et al. (1964) utilized the low glycerol-intermediate rate of freezing technique. They demonstrated that the best results were obtained with 17.5% (W/V) glycerol concentration and 4% sorbital. Equal volumes of packed red cells and 0.9% sodium-chloride solution contains 35% W/V glycerol and 4% sorbitol, were added in a flat stainless steel container which was frozen in liquid nitrogen. They reported in vitro recoveries of 97-98% and 24 hour post-transfusion survival of autologous blood ranged from 94-99%. In 1970 Krijnen et al. improved their original method slightly by freezing the glycerolized blood in a seamless, cylindrical, aluminium aerosol can and obtained excellent results.

Rowe et al. (1968) also utilized the low glycerol liquid nitrogen freezing method for preservation of human red cells for transfusion. They froze a mixture of packed red cells and glycerolizing solution in the ratio 1:1 in a flat stainless steel container by direct immersion in liquid nitrogen. The protective solution was composed of the following:-

Glycerol	28% v/v
Mannitol	3%
Sodium Chloride	0.9%

The frozen blood was stored in liquid nitrogen temperature (-196°C). After thawing and processing more than 90% of the cells were recovered intact and the post transfusion survival was 96%. An attempt to store the blood, that was frozen in liquid nitrogen in a mechanical refrigerator at -80°C gave disappointing results. Rowe in 1971 substituted a plastic polyethylene bag (Haemoflex) for the stainless steel container. The process of freezing, thawing and washing were all performed in the same bag, hence the time necessary for preparation of the unit was markedly reduced.

Jenkins and Blagdon (1971) have adapted Krijnen's technique of blood preservation utilizing the low-glycerol-liquid nitrogen-aluminium canister method. They introduced some modification on the freezing container by using a one piece aluminium bottle but with a push-in rubber stopper "Suba Seal" instead of a crimped on vial cap and screw cap dust seal cover of Krijnen et al. Experiments were performed to determine the optimal final concentration of glycerol that suits the new canister. They found that 20% W/V glycerol and 4% sorbitol gave the best protection. Freezing was performed by immersing the aluminium bottle in liquid nitrogen where the rate of cooling was approximately 0.7°C per second. Blood was then stored in the vapour phase of liquid nitrogen at -150°C.

REMOVAL OF GLYCEROL AND PREPARATION OF THE FROZEN CELLS FOR TRANSFUSION

The use of glycerol as a cryoprotective additive made possible the preservation of human red cells in the frozen state for very long periods, and in fact there are no practical difficulties concerning freezing and storing the erythrocytes in this way. However, the

preparation of these preserved cells for routine transfusion presented difficulties for some time. Although glycerol appeared to be nontoxic when transfused to recipients, as it is an intermediate product of carbohydrate metabolism, the impregnation of the erythrocytes with glycerol makes it osmotically hypertonic so that its administration into the circulation results in rapid swelling and lysis of the cells. For the same reason the cells can not be washed with isotonic solutions (Sloviter 1951a).

While glycerol penetrates the human red blood cells easily, its removal has represented a major obstacle. The rate of water diffusion into the cell is approximately four times greater than the rate of diffusion of glycerol out of the cell. For this reason replacement of red cells equilibrated with high concentrations of glycerol into isotonic solution results in absorption of water much faster than losing glycerol (Tullis et al. 1956).

It seemed that the best way of removing the glycerol out of the red cells was by reversing the process by which it entered while keeping in balance the osmotic pressure between the cells and its surrounding medium. This would be achieved by suspending the cells in solutions with sufficient osmotic pressure to hold the water whilst the glycerol diffuses out of the cells.

In the early days of investigations, glycerol was completely removed from the red cells prior to transfusion by dialysis against saline solutions containing gradually decreasing concentrations of glycerol (Sloviter 1951a). However this method was cumbersome, slow (48 hours) and carried a high risk of bacterial contamination.

Lovelock (1952) described a method for rapid removal of 15% glycerol out of previously frozen-thawed red cells by increasing the osmotic pressure of the suspending medium to a very high level. He found that the addition of 0.33 sodium citrate to the suspending medium will increase its osmotic pressure seven-fold, glycerol and water would thus diffuse out of the cells until an osmotic equilibrium is reached. The cells would then contain only small amounts of glycerol that permitted its centrifugation and resuspension in plasma or any isotonic medium.

Brown and Hardin (1953) have used a stepwise washing technique, as a method of removing glycerol from previously frozen-thawed erythrocytes. Washing was accomplished by serial processes of resuspension, centrifugation and decantation. The washing solution consisted of 0.48 M sodium lactate containing a small amount of glycerol. The Authors also described a continuous washing centrifuge and method for removal of intracellular glycerol using a salt solution of progressively decreasing hypertonicity. Although no in vivo survival data was given, the authors pointed out that this method of washing was superior to the stepwise technique.

The methods of deglycerolization of red cells by washing with sodium citrate or sodium lactate, though successful, were found to be unsatisfactory. These hypertonic solutions result in severe sludging of the red cells on centrifugation, and the subsequent disaggregation gives substantial haemolysis (Sloviter and Ravdin 1965).

Chaplin et al (1956) described a method of glycerol removal by stepwise dilution washing method. The method consisted of four sequential wash cycles in citrate-glycerol solution containing

respectively 16%, 8%, 4% and 2% glycerol in 3% dodium citrate. The cells were then washed three times in unbuffered saline. The whole process required a period of five hours to be completed.

Slöviter (1956) developed a method, for processing thawed erythrocyte-glycerol mixtures, which did not require the complete removal of the glycerol prior to transfusion. Based on the fact that human erythrocytes are much less permeable to glucose than to glycerol, a hypertonic glucose solution was added to the thawed blood-glycerol mixture where it provided an osmotic counter balance to the glycerol within the cells. After standing for 10 minutes at room temperature the mixture was diluted by the addition of twice its volume cold 0.9 percent sodium chloride solution. At the end of this process the resulting suspension contained approximately 4.5 percent glycerol and 5.5 percent glucose in addition to the isotonic electrolyte. It had a haematocrit of 10%, and osmotic pressure approximately four times that of the blood. This dilute suspension may be either transfused directly or centrifuged to separate the red cells which may then be resuspended in saline or plasma before transfusion.

SPECIAL TECHNIQUES OF WASHING

Continuous centrifugation:

The first investigators to make use of the idea of continuous centrifugal washing for the removal of glycerol from the red cells were Chaplin and Veall (1953). The apparatus used was based on the principle of "long traverse centrifuge". The principle of this method was that an upright conical flask was rotated on a turntable at speed sufficient to maintain the red cells at the periphery. The wash solution was administered, through inlet tubes, into the periphery of

the vessel to create a turbulance in the red cell mass. The wash fluid of lower density than the cells, was collected from an ejection outlet near the neck of the flask. Hypertonic sodium citrate and 0.9% sodium chloride were the washing solutions used and the whole process took about 2½ hours.

At the same time Brown and Hardin (1953) in U.S.A. were developing a system based also on the "long traverse centrifuge" in which a standard laboratory centrifuge was utilized. Four units of blood in standard blood bottles were simultaneously washed to get rid of their glycerol content. The washing fluids, consisted of glycerol free salt solutions of gradual decreasing tonicity, were fed from a common reservoir in the centrifuge head into the red cell-glycerol mixture during centrifugation. The red cell mass was kept in the outer position of the container by the centrifugal force and thus prevented from over-flowing with wash fluids. Unequal flow to the four containers resulted in uneven washing of the different units.

In 1958 Mollison et al. designed a centrifuge head to process two units of blood kept in standard M.R.C. glass bottles. In their design they avoided the defect met within the system of Brown and Hardin by providing a separate supply of washing fluid for each bottle, thus insuring even washing of the two units. The whole process took about one hour and a half. That the M.R.C. bottle could be used in that design was important, because it showed that the blood could be collected, glycerolized, stored and processed in the same container and therefore the risk of bacterial contamination was minimized.

Tullis et al 1956 adapted the Cohn Blood Fractionator, a sterile closed system, to add and remove 50% W/V glycerol to units of packed

cells before freezing and after thawing. This apparatus was originally developed for the separation of plasma on a continuous flow basis. The use of a Cohn fractionator for glycerolization and deglycerolization has eliminated the need for excessive manipulation seen in manually processed red cells. Basically, the apparatus consists of an inverted bowl of complex design rotating on a fixed base. The wash solutions are continuously introduced and removed from underneath the bowl. The fluids enter the bowl at its periphery, percolate through the packed cells and are removed proximate to the axis, so that a continuous separation is possible, with the red cells collected in the peripheral portion of the bowl. Deglycerolization was accomplished semiautomatically by continuous flow washing of the red cells with a gradient of hypertonic electrolyte solution made up from:

Solution (1) first wash:	Solution (2) second wash:
Sodium lactate 0.496 M	Sodium lactate 0.156 M
glycerol 10%	Potassium chloride 0.004 M
Potassium chloride 0.004 M	Distilled water Q.S.
Distilled water Q.S.	

The time required to process one unit was 120 minutes utilizing 4 litres of wash fluid, and after completion of the process about 70 to 90% of the red cells were recovered intact, (Tullis et al 1956). Despite the cost and the complexity of the system and the relative slowness of the washing time, the production of sterile unit of blood has made possible post-thaw storage for distribution to other hospitals and massive clinical experimentation (Tullis et al 1958, Ketchel et al 1958, Hynes et al 1960). During the period from 1956 to 1961, 2,250

units of blood that had been frozen with glycerol, and stored for varying periods of time, were processed by the Cohn Fractionator and transfused to 653 patients of all ages (Haynes et al 1962). Also during this period some improvements were introduced to the original method. A third saline wash was suggested by Tullis et al (1958), 0.1% dextrose and 0.008 M $MgCl_2$ was introduced by Haynes et al (1960) and the processing time was reduced to 60 minutes. Although the Cohn Fractionator had successfully operated for about ten years from the stand-point of producing a clinically acceptable product, it was criticized on the basis of expense, slowness and the high in vitro loss of erythrocytes during washing (20%)(Valeri 1970). In addition, the process presented significant difficulties as the different parts of the apparatus have to be demounted, reassembled, sterilized and left to cool for long periods before being remounted again.

A joint project of Tullis and the Arthur D. Little Co. yielded a new pattern of bowl, the A.D.L. reusable bowl, a less expensive version of the Cohn fractionator (Tullis et al 1967). Two types of bowl have been developed, a stainless steel and a rigid clear plastic, both of which could be mounted onto the spindle of a standard refrigerated laboratory centrifuge by a special chuck attachment. As with the Cohn Fractionator, the new system was used for both the addition and removal of the glycerol, to and from the red blood cells. Unlike the old system, the A.D.L. bowl was fed and drained through a sterile rotary seal at the top. The wash solution and details of the procedure were similar to those described by Tullis et al (1958). Utilizing the new system the authors obtained 90 per cent in vitro recovery, and the processing time was reduced to 30 minutes, but the total volume of the wash solution remained as it was with the Cohn fractionator, four

litres (Valeri et al. 1969). Nevertheless, the A.D.L. reusable bowl represented only a transitional step in the evolution of the continuous flow centrifugation procedure, for, it was obvious from the start that the ideal system is that which employs a disposable liner. The time spent in disassembly, cleaning, reassembly and sterilization of the different parts, after each run still represented a major obstacle.

In 1968 Schlutz and Bellamy from the Travenol laboratories developed a disposable cell washing system that utilizes the principle of counter-flow washing. It consisted of a collapsible plastic chambre and rotary seal, both of which were disposable. The system was designed to serve with the RC-3 centrifuge and by slight modification could be fitted into a standard laboratory centrifuge cup. The processing chambre, when fully inflated, was in the form of a cylinder topped at either end with a conical section. The wash fluids enter and leave the bag through an inlet and outlet tubing, the former extends all the way down to the bottom of the bag. The processing bag was constructed in a manner, so that the incoming wash solution creates an extreme turbulance within the red cell mass but without disturbing the cell-supernatant interface which is formed by the centrifugal force. Both the rate of flow and the density of the wash solution were important factors that determined the speed and efficacy of washing. If the flow of the wash solution exceeded a certain rate, the red cells would be forced out of the processing chambre. Similarly, if the incoming solution was greater in density than the red cells, all the cells would be washed out of the vessel. A wash solution lighter than the supernate channelled through the red cell mass, without mixing with it, and travelled to the waste part without

efficient washing. This system was incorporated into a fully automated continuous flow-double unit cell washing machine, Elutramatic TM System, which included a distribution manifold, a roller pump, a control centre, timers and flow rate potentiometers. The system is connected to the external central circuit of an RC-3 centrifuge allowing the automatic operation of the latter. Two units of frozen blood could be simultaneously washed in 70 minutes utilizing approximately four litres of wash solution per unit (Orlina et al 1972).

Meryman and Hornblower (1969, 1972a) introduced an important modification in the process of washing red cells frozen with the high-glycerol-slow freezing technique. They substituted a hypertonic solution of sodium chloride for the glycerol-lactate-saline solution in common use up to that date. After thawing, the glycerolized red cells were first diluted with 150 ml of 12% sodium chloride buffered to PH 7.0,left for few minutes to equilibrate at room temperature then 1000 ml of a solution containing 1.6% NaCl PH 7.0 was added (the pre-wash dilution). The cells were then washed with an additional two litres of saline in a centrifugal-cell washing device; the first litre consisted of 1.6% sodium chloride solution and the second was a solution made up of 0.8% sodium chloride, 0.2 per cent glucose buffered to keep a pH not less than 7.0. Utilizing the above described methods in the ADL centrifugal cell washing apparatus, the authors reported a mean in vitro recovery of 98 per cent, supernatant haemoglobin 24 mg/100 ml and post-transfusion survival ranged between 76 and 90%.

Runck and Valeri (1969), following extensive studies into the variables affecting the washing procedure of frozen blood, came to the conclusion that the initial dilution of the glycerolized blood, prior

to washing, with hypertonic salt solution so as to reduce the glycerol concentration to about 5 percent would permit rapid collection and washing of the cells. The method was applied for both high and low glycerol preserved blood (Valeri et al. 1969). In 1974a Valeri reported a modification of the method of washing previously frozen red cells in the Naval Blood Research Laboratory at Chelsea Massachusetts. Red blood cells that contained 40% W/V glycerol were diluted with 150 ml of 12% sodium chloride solution and left to equilibrate for two minutes at room temperature. The mixture is then diluted with 500 ml 1.6% NaCl solution. This process resulted in a reduction of the concentration of glycerol to about 18%. The cells are then washed in the Elutramatic system with 2.2 litres of isotonic sodium chloride within 30 minutes.

In 1971 Tullis et al. developed a disposable plastic centrifuge bowl and harnesses to replace the ADL bowl. It is made of clear plastic and has the same geometry and basic principle as the reusable ADL bowl. Removal of glycerol is achieved by washing the red cell in a continuous-flow centrifugation system utilizing about four litres of wash fluid (glycerol-lactate-saline). The time required to process one unit is 25 minutes. Experiments with the new system has given a satisfactory result as regards the in vitro recovery (89%) and the immediate post transfusion survival (91%). The new set was later incorporated into a continuous-flow single unit cell washing machine (Haemonetics 10) which is composed of a disposable rigid polycarbonate bowl driven by a compact centrifuge "Blood Processer No.10". The bowl is made up of two compartments one that rotates and another which is stationary. The thawed glycerolized blood enters the continuously spinning bowl from the top via an inlet port, which goes down to the bottom, where it is distributed to the periphery. As the bowl fills

the supernatant fluid separates from the red cells. The flow of the washing solutions is similar to that of the blood and the geometry of the bowl is constructed so that it keeps the cells suspended against the flow of the wash solution. After completion of the washing process the red cells are syphoned into a transfer plastic pack for transfusion. The Haemonetics-10 is relatively inexpensive to purchase but the cost of the disposable bowl and harnesses is high. However it is claimed that two units of blood for the same patient could be processed in the same liner (Valeri 1974a).

REVERSIBLE AGGLOMERATION AND THE HUGGINS CYTOGLOMERATOR

In a search for a simple and an inexpensive alternative method, to the Cohn blood fractionator, for the removal of glycerol from the thawed erythrocytes Huggins (1963a) investigated the phenomenon of red cell agglomeration that usually occurs in aqueous-nonelectrolyte solutions. This phenomenon appears to be due to an interaction between the gamma-globulin of the plasma and the lipo-protein of the red cell membrane at an acid pH between 6.1 and 5.2. As a result of this reaction a reversible complex is produced and when the salt concentration of the medium is lower than 0.02 M these complexes coalese to form a large clump of red cells which sediment rapidly without the need for centrifugation. The phenomenon could be reversed by either the addition of electrolyte to break the gamma-globulin-gamma-globulin bonds or by increasing the pH thus breaking the gamma-globulin-lipo-protein complex. A novel method, circumventing the need for a centrifugation has been developed by Huggins(1963b) who made use of the rapid sedimentation of the red cells in large aggregates to wash them

by discarding the supernatant fluid and resuspending in a clear solution. Successive dilution, and sedimentation, provided adequate washing of erythrocytes that had been frozen with high concentrations of cryoprotectant. When first described the method was applied for processing red cells that have been frozen and thawed in the presence of 8.6 M dimethyl-sulfoxide (DMSO). After three consecutive processes, using four litres of wash fluid of dilution and sedimentation, the DMSO concentration was reduced to 0.2 g percent (Huggins 1963c and 1964). Doubts about the safety of DMSO led Huggins to substitute the nontoxic additive, glycerol in the freezing step (Huggins 1965). Glycerol was used in the same concentration as the DMSO i.e. 8.6 M or in a final concentration of 4.5 M (40% W/V). Initially the process of deglycerolization was performed manually by first diluting the thawed blood with 500 ml of 50% dextrose in water, followed by 3 litres of 8% glucose and 1% fructose. After sedimentation, the red cells were separated by decanting the supernatant and the dilutional wash was repeated once more. The agglomerated red blood cell mass was then resuspended by the addition of 250 ml of isotonic saline. The whole process utilized 6.75 litres of fluids and required nearly one hour to prepare one unit of blood for transfusion. However with the introduction of the cytoglomerator, a specially designed automatic processor and its disposable blood freezing unit in 1964, five units of blood could be processed simultaneously in 20 minutes. This was considered a revolutionary event in the history of preservation of blood by freezing particularly if compared with the slow complicated Cohn fractionator. By 1965, 1081 units of blood had been frozen and stored at -85°C for up to 18 months, then thawed, processed by the Huggins method and transfused to 124 patients with an average of about

9 units per patient. With the exception of 136 units all the blood has been frozen with 8.6 M glycerol (Huggins 1965). Huggins reported an in vitro recovery of about 90% and post transfusion ^{51}Cr survival from 87 to 95% after 24 hours. Furthermore he pointed out that this method of washing had eliminated the isoagglutinins from the processed blood and removed the white blood cells, extracellular potassium, citrate anticoagulant and plasma.

Because of its simplicity, Huggins' method, has been extensively used for clinical application at the Naval Blood Research Laboratory in Chelsea, Mass.(Valeri 1966). A positive Coomb's test was observed with the erythrocytes that have been washed by this method. This appeared to be due to firm coating of the cells with some component of the complement system and was inhibited by the addition of 0.3% sodium EDTA; to the glycerolizing solution (Huggins 1965). However, the addition of EDTA was found to have an adverse effect on both the in vitro recovery and in vivo survival of the red cells. Thus red blood cells frozen with high glycerol EDTA solution showed a mean in vitro loss of 25% and 24-hour post-transfusion survival about 70% (Valeri 1970).

Whether EDTA was added to the glycerolizing solution or not, processing of the thawed glycerolized erythrocytes by Huggins technique, with non-electrolyte solution, resulted in a substantial decrease in the intracellular potassium content and high residual free haemoglobin (Valeri et al. 1969). The decrease in the intracellular potassium was believed to be responsible for the low 24-hour post-transfusion survival (Valeri and Runck (1969a). Further deterioration of the in vivo survival was observed when the red cells that have been frozen

without EDTA were stored at -80°C for longer than 2 years or with EDTA for longer than 1½ years and then processed by Huggins' technique.

By contrast to the Huggins' methods, washing of these prolonged stored cells with electrolyte solution in a continuous flow centrifugation system gave a higher, in vitro recovery, intracellular potassium and in vivo survival and lower free haemoglobin in the supernatant (Valeri and Runck (1969a). The implication of this is that Huggins' preserved red cells should not be washed by the reversible agglomeration technique if it had been stored for more than two years in the absence of EDTA or more than 1½ years with EDTA. A better technique of washing is by an electrolyte solution in a continuous-flow centrifugation system. This has decreased the interest in the reversible agglomeration technique and the cytoglomerator as a means of washing thawed glycerolized red cells. However an outstanding character of the method is the rapid output of units, a matter of major importance if a large number of units are required for surgical use.

OTHER PROTECTIVE COMPOUNDS

Since the protective action of glycerol was found to be due to a colligative property rather than to a specific character of the compound itself, it was to be expected that a similar effect would be provided by many other compounds.

Lovelock in (1954) had tested 15 neutral compounds, mostly with multiple hydroxyl groups, including mono-, di-, and poly-hydric alcohols, amides and sugars. The compounds were classified into three categories penetrating, partially penetrating and non penetrating. He found that only non-toxic neutral solutes which have a low molecular weight and which can easily permeate the red cells would afford full protection. However, Glauser and Talbot 1956 found that permeation of the red cell is not a prerequisite for producing complete protection as polymers with high molecular weight were also found to be effective in this respect. Instead they ascribed the protective action of any additive to a specific character in its structure where a polar carbon to oxygen, bond is present with the oxygen available for bonding with the hydrogen of the water molecule of the solvent.

Sloviter (1958) investigated the action of polyethylene glycols, high molecular weight polymers, in protecting the red cells against freezing injury. The molecular weight of the compounds tested varied from 500 to 1500. However, all of them were found to be effective with an optimum concentration of 25% by weight, a finding which conflicted with the colligative action found by Lovelock.

Doebbler and Rinfret (1962) investigated 27 compounds of various chemical structure and molecular weight for their protective effect

against the injury of rapid freezing. Included in this list were alcohols, glycols, glycol derivatives, sugars, amino acids, peptides and carboxylic acid salts. They found that, in contrast to Lovelock's results with slow freezing, disaccharides which are nonpenetrating compounds afforded greater protection than most of the monosaccharides to which the red cells were permeable. In addition they examined the protective action of 14 high-molecular weight polymers, all of which were found very effective. They concluded that the degree of protection afforded by any compound is related to the concentration of potential hydrogen bonding groups in its structure.

However, most of the compounds rested were abandoned either because they are toxic to the red cells in the doses required for protection or produce untowards side effect in the recipient. The only compound that gave results comparable to those of glycerol was the dimethylsulfoxide (DMSO). The protective action of this compound was first discovered by Lovelock and Bishop in (1959) when they found that on a molar basis, DMSO afforded the same protection from freezing to both bovine and human red blood cells. However DMSO offered an outstanding advantage over glycerol. Being of lower molecular size DMSO crosses the cell membrane more readily, and thus the difficulties met with in removing the glycerol from within the cell were greatly reduced. Furthermore, cells which are impermeable to glycerol, bovine red cells, were found to be permeable to DMSO. Most of the experience with DMSO comes from the work of Huggins who in (1963a) developed a method of blood preservation in the frozen state by freezing equal volumes of packed red cells and solution containing 8.6 M DMSO, 8% glucose and 1% functose. A total of 133 units of

blood have been frozen, with DMSO stored at -85°C, for different periods processed by Huggins technique and given to patients without untowards effects. However doubts about the safety of DMSO led Huggins (1964) to shift to the non-toxic, penetrating additive, glycerol.

So far glycerol remains unchallenged as the most nearly ideal cryoprotective agent and all methods in current use today employ glycerol in different concentrations.

RAPID FREEZING

Meryman in 1956 indicated that successful preservation of blood by rapid freezing requires:-

1. A very rapid rate of cooling
2. Storage at low temperature to prevent recrystallization
3. A very rapid rate of thawing
4. Suitable geometry of the specimen to allow uniform heat exchange throughout its surface. Luyet in 1949 fulfilled this requirement by freezing a very thin smear of ox-blood on a glass cover by direct immersion in liquid nitrogen.

In 1955 Meryman and Kafig proposed that the most suitable shape for heat exchange is the sphere or droplet form. In order to attain maximum heat transfer rates they produced a very small sized droplet by passing the blood through rapidly oscillating capillary jet. The droplet size ranged from 0.45 to 0.9 mm in diameter and were sprayed directly onto the surface of liquid nitrogen where they floated for a while and then sank to the bottom. Thawing was performed by transferring the frozen particles to a container of plasma or isotonic

saline at 42°C. Initially 50% intact cells were recovered by this method. However, when whole blood was modified by the addition of 7% glucose the recovery rose to 97%. Two autologous transfusions in humans were performed, and the transfused cells were labelled with radioactive ^{51}Cr. It was shown that the twenty-four hour post-transfusion survivals were 82% and 86%. It was hoped, after this experiment that whole blood could be frozen in large volumes, thawed and transfused without the need for post-thaw processing which is a major drawback of the methods utilizing glycerol. However, it was found later that glucose does penetrate the red cell membrane in amounts sufficient to cause osmotic imbalance in the same way as glycerol (Meryman 1968b). Moreover, the procedure was rather tedious, time consuming and difficult to operate under sterile conditions. In spite of these difficulties the Linde Company (in 1956) adopted the method and designed an apparatus for freezing and thawing blood in pint quantities by the spraying technique under aseptic conditions. Although the device was technically satisfactory it has been abandoned because it was found to be very expensive, bulky and complicated (Meryman 1964 - Blagdon 1972).

The droplet-spray technique was largely abandoned when Strumia et al. (1958a) developed a method for freezing a whole unit of blood, treated with a mixture of glucose and lactose, in a closed metal container. Equal volumes of citrated blood (ACD) and sugar solution were mixed together to give a final concentration of 0.2M lactose and 0.7M dextrose. Freezing was accomplished, in a flat sealed metal container, 3-4 mm in diameter, by immersion in dry-ice-ethanol mixture at -78°C blood was thawed in a water bath at 37°C. With this method 95% of the frozen cells were recovered intact. In five human

transfusions of blood labelled with ^{51}Cr frozen and thawed without being stored the 24-hour post-transfusion survival was 2% less than autologous fresh blood. Further experiments (Strumia 1960a) showed that in vitro recovery of the cells remained unchanged for two years at -93°C while the 24-hour post-transfusion survival of this blood was similar to that frozen and thawed immediately without storage. It was also clear that storage at -58°C and -70°C were unsatisfactory for long periods, although the latter temperature was adequate for 3-4 weeks (Strumia et al. 1960b). Freezing in a closed container was quite promising because it offered major practical advantages, although in unit-volumes the heat exchange was less efficient than with the droplet-spray method and necessitated the use of a substantially higher concentration of sugars. In spite of this, the technique was soon adopted by Linde Co. group (Rinfret 1963) who developed a corrugated aluminium container for freezing a whole unit of sugar-modified blood. The container was filled only up to half capacity with blood so that on freezing, shaking the container would spread the blood over the entire inner surface as a thin layer. Further improvement in heat transfer was achieved by covering the outer surface of the container with an insulating layer of PVP (Rinfret 1963). Blood frozen by this technique was stored in liquid nitrogen while thawing was carried out by continuous shaking of the container in a water bath at 45°C. Linde Co. designed a sophisticated apparatus to perform all these steps (Hurn 1968). In this way, the advantages offered by rapid-freezing were obvious, for a unit of blood could be collected directly into the freezing container, frozen, stored in liquid nitrogen and prepared for transfusion without post-thaw washing, in less than five minutes. Although the system seemed very promising,

practical application brought about the discovery of its disadvantages (Blagdon 1972). First it was shown that freezing red-cells in the presence of glucose, render them osmotically unstable, so that on transfusion they rapidly haemolyse (Bloom et al. 1962). Moreover, the amount of free haemoglobin present in the supernatant was high (Blagdon 1972).

When glucose was abandoned as a cryoprotective additive, lactose was introduced (Strumia et al. 1958a - Rinfret 1963). Certainly lactose does not penetrate the red cell membrane (Rinfret 1960), but because of its low molecular weight it is osmotically active and may cause substantial increases of the recipient's plasma osmotic pressure. Experimental work with dogs and rats showed that lactose, in doses required for protection, produced pathological lesions in the kidneys, lungs and livers of the animals. This suggested that if lactose, or any other low molecular weight additive, were to be used as cryoprotective agents, they must be removed prior to transfusion (Pert et al. 1964).

After the rejection of all sugars as cryoprotective additives, attention was directed towards substances with high molecular weight in the assumption that these compounds would have little effect on the osmolarity of the recipient's plasma. Previously many investigators have reported on the cryoprotective activity of many solutes. Of special interest was the finding of Bricka and Bessis (1955) that polyvinylpyrolidone (PVP) and dextran protected human red-blood cells against freezing injury. Both substances are high molecular weight, pharmacologically inert, polymers which are incapable of penetrating the cells. In their experiments the authors added one volume of

packed cells to four volumes of additive in a 15 mm diameter aluminium tube. Freezing was achieved by immersion in dry ice-alcohol mixture. A recovery of 95% was obtained in the presence of 40% PVP in saline and at cooling rate of $1^{o}C$ per second.

Sloviter in 1958 has also reported on the cryoprotective activity of polyethylene glycol, a polymer with a M.W. in the range of 500 to 1500, when it was added to red-blood cells in a final concentration of 25% by weight. Looking into the mechanism of the additive's protection, a group of investigators, at Linde Research Laboratories tested a variety of compounds for their cryoprotective activity. Among the list was, the plasma volume expander, PVP whose activity in this field was previously demonstrated in 1955 (Bricka & Bessis). Of all the compounds tested PVP showed an outstanding ability to protect red-blood cells against rapid-freeze-thaw injury. At about equal weight concentration PVP and glycerol offered similar protection at a cooling rate of $3^{o}C$ per second (Doebbler & Rinfret 1959 - Doebbler et al. 1960 - Doebbler and Rinfret 1962). Using rabbit blood Doebbler et al. (1961) reported a recovery of 87 to 96% after freezing a mixture of ACD-blood and 7% PVP "final concentration" in small aluminium tubes. On transfusion 85 - 98% of the transfused cells survived in the circulation after the first 24 hours.

By this time it has been realized that PVP was the most efficient extracellular additive that could protect the red-cells against the injury of rapid freezing (Rinfret et al. 1962). Accordingly most of the subsequent work, which was devoted to the development of a clinically acceptable technique for blood preservation by rapid-freezing relied on the cryoprotective activity of PVP.

Following this experimental work the technique was adopted by the Linde group. Using the Linde Automatic Blood Processing Unit Richards et al. (1964) reported a recovery of about 96% and 24-hour post-transfusion survival of 80% for blood frozen and thawed in the presence of 7.5% PVP. In a series of 30 transfusions of whole units, no instance of serious transfusion reaction was observed. Again no incidence of renal function disturbance was recorded, although in their initial trials several patients had dark-coloured urine for 12-24 hours and some showed a fairly high in vivo haemolysis.

Transfusion of up to full-unit volumes of this blood resulted in in vivo survival of approximately 85% of the cells 24 hours after infusion (Sakaida et al. 1965). Because of its large sized molecules (M.W. range from 25,000-40,000) PVP, does not permeate the cell or affect the osmolarity of the plasma after transfusion. But, unfortunately, it is this high molecular weight that evoked a problem concerning its use. Since this substance is not metabolized or disintegrated into smaller sized molecules to permit its excretion, the compound is stored in the reticuloendothelial system (R.E.S.) for very long periods (Altemeier et al. 1954). That the large molecular weight grades of PVP are retained in the body for very long periods was also suspected by Bloom et al. 1962, 1965 Richards et al. (1964), and Knorpp et al. 1967, and confirmed by Pert et al. 1965. The prolonged retention of PVP by the R.E.S. is probably a disadvantage which must be considered seriously. Another disadvantage of PVP-frozen blood was the high level of free haemoglobin in the supernatant after thawing (Valeri 1970 - Blagdon 1972). A post-thaw washing was suggested, (Meryman 1968b, Valeri 1970), as a possible solution for

removing the PVP, and the excess free haemoglobin. However, the proposal did not receive great acceptance and it has been continuously argued that this step would deprive the method its only advantage. Moreover, washing erythrocytes processed by this technique usually resulted in extensive haemolysis (Bloom et al. 1965). Finally if PVP frozen blood is to be washed, one might find it much easier to use glycerolized-frozen blood as the latter is definitely nontoxic, does not need special equipment for processing and controlled rates of freezing and thawing are not required. Above all it yields a clinically acceptable product (Pert et al. 1965 - Meryman 1968b). The possibility that PVP is potentially toxic, has led investigators to seek other polymers which might be more clinically acceptable. Dextran, (M.W. 40,000), in a concentration, of 15-21 g/100 ml and albumin in a concentration 17-22 g/100 ml were found to possess cryoprotective activity. However, red cells frozen in their presence survived poorly when transfused into human volunteers (Strumia and Strumia 1964).

The assumption that PVP retention might be circumvented by using a low molecular weight fraction of PVP was first suggested by Pert et al. (1964). Low molecular weight PVP was obtained by chromatographic fractionation of "K-22" PVP (average M.W. 25,000) on G-75 Sephadex columns. No apparent difference in activity was noticed between different fractions, the average red cell recoveries varied between 95 and 97%. Although the idea seemed good and has been tried at the Linde Laboratories, no successful reports have been published (Hurn 1968).

As a further substitute for PVP, Hydroxyethyl starch (HES) has been suggested (Knorpp et al. 1967). Similar to PVP and dextran,

HES, is a plasma volume expander used as 6% solution, but unlike PVP it is slowly hydrolized in the circulation into its precursor, glucose, by the plasma amylases. Thus its transfusion does not present the problem of long term retention experienced with PVP. For freezing, Knorpp et al added whole blood to a solution of HES in saline to give a final concentration of 15% HES. The mixture was frozen in liquid nitrogen with continuous agitation using the Linde Automatic Blood Processing Unit. Upon thawing 97.4 percent of the red cells were recovered intact. Thus HES was as effective as PVP and, offered the advantage of being gradually metabolized by the body. The average M.W. initially used was 450,000.

The efficiency of HES in protecting red cells against injury of rapid freezing was confirmed by Robson in 1969. He reported an in vitro recovery over 96% by this method. The method of freeze preservation of the blood by HES was slightly modified to employ packed red cells instead of whole blood (Knorpp et al 1971). Packed erythrocytes were added to HES - saline solution to give a final concentration of 14% of HES. Although HES is considered non-immunogenic and nontoxic (Maurer and Bererdinalli 1968) infusion of such a highly viscous mixture may represent a potential hazard to the transfused recipient. In the first instance because of its high viscosity, HES-erythrocyte mixtures gave severe difficulty and delay in transfusion, besides its presence in the circulation might encroach upon the renal haemodynamics (Stella Baar 1973). Secondly the introduction of a mixture with such a high viscosity might interfere with blood coagulation mechanism and results in bleeding diathesis (Thompson & Gadsden 1965, Lee et al 1968). Thirdly because of the post-thaw

unstability of erythrocytes processed by this method, the free haemoglobin content of such blood is very high (Blagdon 1972). Fourthly red blood cells, preserved by this technique lose 87% of its intracellular K+ to the surrounding medium (Meryman (1971c), a matter which might affect its in vivo survival (Valeri and Runck 1969a) and increase the extracellular K+ concentration to an unacceptable level.

In spite of the fact that in vivo studies in monkeys showed no adverse effect of HES-frozen blood on the blood coagulation, reduction of the viscosity of the HES-blood suspension was advised as an additional safety measure. As a method of lowering the viscosity, HES can be washed out of the erythrocyte-HES mixture without compromising the biological value of the product (Knorpp et al. 1971). In so doing the authors claimed a substantial improvement in the post-thaw stability of the cells in saline besides the complete removal of the excess haemoglobin and potassium.

Instead of washing, the HES could be diluted to the required degree by the addition of a physiological salt solution to the thawed blood. This reduces the concentration and hence the viscosity of the HES in the blood to limits which permit its transfusion without difficulty, (Stella Baar 1973). An alternative approach to avoid transfusion of HES of high viscosity is to use low molecular weight starch (Weatherbee et al. 1974). Preliminary experiments showed that this form of starch provided even better protection to the red cells than the high molecular, plasma expander type.

It has always been emphasized that the ultimate aim in blood preservation by rapid freezing is the production of a preparation that, after thawing, require no further processing. Verification of

this ideal one-step method, would have eliminated the need for the exhaustive post-thaw washing which is demanded by the glycerol technique. However none of the above described methods has achieved this aim. Post-thaw processing has been a necessary step either to remove the product of haemolysis or the undesired additives or both. The acceptable limits of these substances represent the major obstacles opposing the development of rapid-freezing techniques. Excessive haemolysis results from the use of low concentration of additives, while higher concentration of the latter brings about osmotic problems.

THE LYTIC MECHANSIM OF RAPID FREEZING

Meryman (1956) defined rapid freezing as the rate at which intracellular ice crystals are produced. When the rate of cooling is rapid, the tendency for preferential extracellular nucleation ceases, and nucleation takes place throughout the specimen leading to ice crystal formation that are predominantly intracellular. Mazur in 1968 and 1970 explained the sequence of events which leads to this result. Exposing the cells to subzero temperatures results in the formation of ice, first outside the cell, at the same time water inside the cell tends to supercool. Because the vapour pressure of the supercooled water is higher than that of the external ice, a vapour pressure gradient is produced which then dictates a process of equilibration. The method of equilibration depends mainly on the rate of cooling. Thus, if this rate is slow, intracellular water will have sufficient time to flow out of the cell and added to the external ice. On the other hand if the specimen is cooled rapidly equilibration is restored by solidification of the intracellular water

in situ. In the latter case ice crystals are formed as a result of homogeneous nucleation, where the nuclei are created from aggregation of the solvent molecules themselves (Meryman 1956).

MODE OF INJURY

Several theories have been put forward to explain the nature of damage and/or the mechanism of protection afforded by rapid freezing.

(i) Ice crystal formation:

Whereas, the presence of extracellular ice appeared to be insignificant (Lovelock 1953a, Meryman 1956, 1963), intracellular one has to be considered seriously. If these crystals are allowed to grow, and they have the liability to do so, beyond the size of the cell that contains them the outcome is obvious. It needs very high rates of cooling to create intracellular ice (Meryman 1956) and when this is reached red cell haemolysis is massive. However it is not known whether the intracellular ice injure the cell during the process of freezing or on rewarming when migratory recrystalization tends to occur. Luyet et al. (1963) were of the latter opinion. They consider that, in the absence of any protective agent, there occur an optimal cooling rate, above and below that rate haemolysis increased rapidly. As regards the cause of haemolysis at the supra-optimal rate, the authors referred it to the formation of intracellular ice during freezing which undergoes recrystallization during warming. Meryman (1963) considered that very rapid freezing leads to the formation of intracellular ice, and thus it is uniformly lethal to mammalian cells. Mazur in (1968 and 1970) has clearly identified the rate beyond which

intracellular ice formation appeared. He indicated that without the addition of additive, maximum red-cell recovery occurs at a cooling rate of 3000°C per minute. Intracellular crystallization of water starts at rates of 5000°C per minute and this coincides with the rates at which red cell recovery declines. He also suggested that cooling rates below-optimum exposes the red cells to the effect of high salt concentrations.

As regards protection, no additive has yet been discovered to check the deletrious effect of intracellular ice formation (Hurn 1968).

(ii) Salt Concentration:

Whatever the rate of cooling might be, as freezing proceeds gradual diminution of the liquid-water occurs both inside and outside the cell. This exposes the cells to the deletrious effect of increased concentration of intra and extracellular salts. Since the latter prcduces its effect through a bio-chemical reaction, the rate of this reaction shows both time and temperature dependence (Meryman 1956). It follows that, at a relatively higher range of temperature, rapid freezing allows no sufficient time for the concentrated salts to produce its effect. As the temperature drops the rate of the chemical reaction ceases and comes to a halt below certain temperature level.

Protection against salt concentration effects required an agent capable of penetrating the cell membrane.

(iii) Dehydration:

That dehydration beyond a certain level is a primary cause of injury has been suggested by Doebbler and Rinfret (1962) Rinfret 1963

and Meryman (1967).

Rinfret in 1963 demonstrated that as high as 90% of the freezable water in the specimen can be converted into ice and still give little haemolysis. However any water removed beyond that results in massive haemolysis. Rinfret's experiment did not only show the importance of cellular-hydration, but also demonstrated that intracellular ice formation is insignificant until the ultimate stages of phase transition. Luyet and Kroener (1967) showed that freezing of the blood slowly to $-3^{\circ}C$ removes 80% of the liquid-water without lysis, at $-8^{\circ}C$ 92% of the water is removed but haemolysis is progressive. Rapid freezing through the same temperature reduces the haemolysis. The authors interpreted these results as the fraction of liquid-water between 80% and 92% is critical and if sufficient time is allowed for its transformation, lysis of the red-cells occurs. Rapid cooling protects the cells by giving insufficient time for the freezing of the last fraction of water. Red cells injured from this cause could be protected by the addition of extracellular cryoprotective agents which can hydrogen-bind the vital fraction of water and stabilize the surface hydration of the cell. (Doebbler and Rinfret 1962, Hurn 1968).

(iv) Adenosine triphosphatase (ATP-ase) activation:

Studying the biochemical changes in the previously frozen-thawed blood, Rinfret (1963) observed an abnormal activity of the ATP-ase and choline-esterases which suggested damage to the cation exchange mechanisms of the erythrocytes. Takehara and Rowe (1968) have also investigated the conditions of freezing that increases the activity of ATP-ase in the red cell-membranes to explore the mechanism of

freezing damage. They found that the faster the cooling rate, the more the activation, and that many cryoprotectives could inhibit this activation. They concluded that the rate of cooling is more important factor in the activation of erythrocyte membrane ATPase activity than the absolute temperature attained. They also suggested that activation of membrane ATPase might be casually associated with the change in cation and electrolyte permeability together with the haemolysis that takes place when red cells are frozen in the absence of cryoprotective additives.

MECHANISM OF EXTRACELLULAR CRYOPROTECTION

With a slow cooling rate at a few degrees per minute, high concentrations of glycerol provide good protection to the red cells against freezing damage (Smith 1950). Glycerol, and similar small molecular weight additives, appear to act colligatively, by buffering the concentration of salts in equilibrium with ice at any given temperature above the eutectic point (Lovelock 1953b, 1954).

Luyet in 1949, found that, in the absence of any additive, very rapid cooling rates at hundreds of degrees per second would protect the red cells against haemolysis. Further improvement in recovery was achieved by the addition of sugars (Meryman and Kafig 1955) and polymers (Bricka and Bessis 1955). At a less rapid cooling rate, several degrees per second, sugars and polymers were still effective in preventing haemolysis (Strumia et al. 1958a, 1960a, 1960b, Doebbler and Rinfret 1959). The mechanism by which these additives produce its action was ascribed to its effect in slowing the rate of

ice crystal growth (Lusena and Rose 1956). The nature of this effect has not been definitely identified but it might be connected to the property of these compounds to be strong hydrogen-bonders (Glauser and Talbot 1956, Strumia et al. 1960a, Meryman 1956, Lusena and Rose 1956). All compounds found effective in inhibiting freeze-thaw haemolysis have a polar carbon to oxygen bonds. The oxygen atom is also available to bond with the hydrogen of the water molecule (Glauser and Talbot 1956). At moderate cooling rates, good correlation between the concentration of potential H-bonding groups and the degree of protection has been observed by Doebbler and Rinfret (1962). In addition to binding water, H-bonding sites, also form and stabilize an extended region of oriented water around each molecule. It seems that such solutes would act by preventing completion of the phase transition which normally takes place during freezing (Rinfret 1963, Rinfret et al. 1964, Doebbler and Rinfret 1965). Under these conditions the water will be available as solvents for the salts but does not contribute in the formation of ice crystals. However, Meryman (1971a) has not been able to find any support for this assumption. On the other hand he observed that metastable state of water occurs only at supra-optimal rates of freezing at which complete haemolysis of the red cells takes place. Meryman suggested that at optimal rate of freezing all the "freezable" water has crystallized and the system was in equilibrium. Since the cells under these conditions must have been exposed to the full salt concentration predicted, their recovery means that avoidance of the effects of these salts rather than failure of the solute to be concentrated is the cause of recovering the cells intact. Moreover, after rapid freezing,

the recovered cells showed a great change in electrolyte content which suggested that their recovery was due to temporary reversible alteration in cell permeability. The latter allowed "influx and efflux" of solutes as a result of stresses which would otherwise burst the erythrocytes. That such cells could survive after transfusion is an evidence for the reversibility of these changes (Meryman and Hornblower (1972b). Since a high percentage of red cells could be recovered by very rapid freezing, in the absence of any additives, the cryoprotective agent is, thus, not necessary for the establishment of reversible membrane leak, however, it serves to decrease the rate of cooling necessary to obtain maximum recovery and stabilizes the cell membrane component against the irreversible post-thaw changes.

Another concept of the mode of action of the extracellular additives has been advanced by Rinfret (1963) and Rinfret et al. (1964). It was postulated that extracellular additives are adsorbed to the red cell surface where they surround sensitive sites in the cell membrane and protect them from the deletrious effect of salt concentration. Greiff and Seifert (1968) developed a membrane adsorption test for determining the effect of addition of cryoprotective compounds on the cryosensitive, mucopolysaccharide, sites at the surface of the membrane of chicken red-cells. They indicated that the mechanism of protection seemed to be through the external binding of these compounds to the sensitive sites of the cell membrane.

FREEZING AND THAWING PARAMETERS

I. FREEZING

A. HEAT TRANSFER:

In order to freeze a biological specimen it has to be immersed in a cooling bath which has a temperature considerably lower than -50°C (Cowley et al. 1961). However, to maintain a constant temperature, in the bath, one has to choose a cooling liquid with a boiling point below this temperature. A great variety of natural liquids are available e.g.

Helium	-270°C	Argon	-185°C
Hydrogen	-252°C	Oxygen	-183°C
Neon	-245°C	Methane	-161.5°C
Nitrogen	-196°C		

Of all these fluids, liquid nitrogen has gained the greatest popularity because it is easily available at relatively low cost and is safe to use. However, because liquid nitrogen is only available at its boiling point, its use in cryobiology has been limited by the formation of a thermally insulating layer of vapour around the immersed object (Cowley et al. 1961, Meryman 1956).

When a warm object is immersed in liquid nitrogen several stages exist in the transfer of heat from the solid surface to the cold liquid. First heat is transferred from the immersed object to the liquid and causes it to boil. Because the initial temperature difference is large, boiling is very vigorous and results in the generation of a stable insulating layer of vapour around the solid object. The low thermal conductivity of this layer causes severe

reduction in the rate of heat transfer "film boiling" (Luyet 1961). Heat is transferred during this stage by radiation (Cowley et al. 1961). Secondly, as the temperature difference is decreased, the rate of heat transfer is slowly increased as a result of breaking the continuous layer of vapour into bubbles of gradually decreasing size (Luyet 1961). The maximum theoretical heat flux occurs at the "nucleate boiling" point at which the temperature difference between the solid surface and the coolant is 3.5°C (Cowley et al. 1961). Further decrease in the temperature difference results in reduction in the nucleation sites and hence of heat transfer. Initially at this stage vapour bubbles were forming turbulance in the liquid and therefore rapid heat flux, however the rate of heat transfer rapidly diminishes as the temperature difference decreases below 3.5°C.

As regards freezing of blood, the range of temperature during which rapid cooling is optimal lies between -3°C and -40°C (Lovelock 1953a). However, the rate of cooling within the material depends mainly, on the rate of heat transfer, which is in turn largely determined by the type of boiling at the surface (Luyet 1961). "Film" boiling leads to slow heat transfer and "nucleate" boiling to rapid transfer and hence rapid cooling rate.

The application of thin layers of thermally insulating materials, to the outer surface of the metal container, results in a remarkable increase in the cooling rate when the sample is immersed in liquid nitrogen (Cowley et al. 1961). The surface coating serves to decrease the surface temperature rapidly to the range of nucleate boiling where the heat transfer is maximum, cooling then proceeds at this higher rate of heat transfer. A wide range of organic materials could be

used as surface coating. Cowley et al. have used vaseline and glycerine but they also mentioned that the addition of a powder on the insulating coat further increased the cooling rates. After the development of the blood freezing system at Linde Company PVP in methanol was found to give the best results when the metal container was dipped into that mixture immediately prior to freezing (Rinfret et al. 1962). It should, however, be pointed out that the method of improving the heat transfer by the application of surface coating is effective only with boiling liquids (Cowley et al 1961).

B. AGITATION

Methods of preservation of blood in bulk volumes by rapid freeze-thaw techniques was first introduced by Strumia et al. (1958a) and was adopted later by the Linde Co. and have since been used by many investigators. Blood was frozen in a thin walled metal container of high thermal conductivity. The problems facing the heat transfer were mathematically reported by Rinfret in 1960. He indicated that in cooling any liquid in an aluminium tube the transfer of heat is impeded by four separate barriers which are, from inside outwards,

1. The liquid core in the centre
2. The crystalline ice layer
3. The container wall
4. The vapour layer surrounding the container

If we consider the resistance of the aluminium wall to transfer of heat as one unit, then, during freezing the highest resistance is offered by the vapour layer and the unfrozen core in the centre. Since reduction in one of the greatest insulators would most effectively reduce the total resistance, then reduction of the resistance of the

vapour laver to one tenth of its value would lower the total resistance by about 40%. As we have seen previously the resistance of the container - coolant interface could be reduced by coating the outer surface of the container with thin thermally insulating material (Cowley et al. 1961). However, it is not sufficient to accelerate the cooling rate only at the interface between the container and the coolant liquid. Further increases in the cooling rate could be achieved by agitation of the container, a process which promotes convection (Strumia et al. Strumia 1958b, 1960a, Rinfret 1963, Sakaida et al. 1965). Acceleration of the rate of heat transfer to high degrees are not always benificial, as extremely rapid rates of cooling are destructive.

II. THAWING

It is assumed that the use of cryoprotective agents would minimize most of the hazards produced by freezing or thawing so that neither rapid cooling nor rapid warming is essential. However, there is ample evidence in the literature that even in the presence of additive, rapid rates of thawing are beneficial.

Thawing of blood, which has been previously frozen in aluminium tubes proceeds much more slowly than the freezing process (Rinfret 1960). It took the phase transition front approximately double the time during thawing, to reach the same distance travelled during freezing. This is due to the fact that latent heat has to be conducted through the liquid sector which has the greatest thermal resistance under these conditions. The important point in this case is that the solid central core, because of its relatively high conductivity,

will reach an equilibrium at a temperature just below the melting point. This means that the red cells, in this region, will be held in the critical range of temperature all the time the phase transition boundary is proceeding towards them. Rinfret (1960) calculated that a reduction of the liquid resistance by 50% would decrease the total thermal resistance by 40%. He suggested that this could be achieved by continuously shaking the container during thawing, a matter which allows transmition of heat via convection, rather than conduction, and thus cut down the time of thawing.

The advantage of agitation of blood was first reported by Strumia et al. (1958b). Agitation of blood during freezing and thawing resulted in much higher recoveries and allowed the use of containers with a larger cross-section or the use of lower concentrations of additives (Strumia et al. 1958b, Rinfret 1963). than when no agitation was employed. Shaking during thawing is more important than during cooling, although the latter has the advantage of spreading the blood all over the entire surface of the container, especially when it is partially full, and thus reduce the distance through which heat has to travel (Hurn 1968). There is a positive correlation between the red cell recovery and the frequency of shaking, however, the relation is not absolute as excessive shaking can bring about mechanical damage to the cells (Rinfret 1963).

COOLING RATE

Study of the relationship between cooling rate and red cell recovery have shown that there is a certain range of cooling rates at which maximum recoveries are obtained (Luyet et al. 1963, Rapatz et al.

1968). This effective range, however, varies according to the presence or absence and to the nature of the additive. Thus without any additive maximum recovery was obtained at cooling rates of 1600°C to 3500°C per minute. Some additives like glycerol prevent haemolysis at low cooling rates. At 10% concentration its maximum effect is seen at a range of temperature from 5°C to 150°C per minute whereas at rapid rates of cooling it is not only ineffective but actually exerts a damaging effect. Other additives e.g. dextrose are effective only at high cooling rates of the order of 2,500 to 3,500°C per minute. Cooling rates above and below these ranges produces excessive haemolysis. Optimum rates of cooling are the result of interaction of the two types of freezing events. High salt concentration produces damage of the red cells at sub-optimal cooling rates and intracellular ice formation at cooling rates above-optimal (Mazur 1970). The optimum rate can therefore be defined as that rate which is "slow enough to prevent production of intracellular ice and yet is rapid enough to minimize the length of time during which cells are exposed to salt concentration effects" (Mazur 1970).

In the range of slow and moderate rates of cooling an increase in the cooling rates causes an increase in the red cell recovery and improves the level of protection afforded at a given additive concentration (Doebbler et al. 1960, Doebbler and Rinfret 1965). At slow cooling rates, a considerably higher concentration of additive is necessary, in order to obtain good red cell recovery (Doebbler et al. 1960, Rapatz et al. 1968). On the other hand the protection afforded by many additives decreases rapidly to nil and may even become negative at increasing cooling rate (Luyet et al. 1963).

STORAGE

For successful preservation, rapidly frozen blood, should be stored at temperatures considerably lower than that at which recrystallization of ice could take place or salt concentration could produce its effects. There are many reasons to believe that (-79°C), temperature of dry ice, is unsatisfactory temperature for long-term storage (Rinfret 1963, Meryman 1963). Ice crystals are still able to grow at temperatures as low as -70°C (Strumia et al. 1960b). Although, all the evidence indicates that temperatures in the order of -93°C (Strumia et al. 1960b) or -100°C (Meryman 1963 - Rinfret 1963) are cold enough to maintain the viability for periods of up to two years, most cryobiologists preferred to employ liquid nitrogen refrigerators. In its liquid form, nitrogen has a temperature of -196°C while the temperature in the vapour phase is approximately -150°C. Liquid nitrogen is easily obtainable, safe and inexpensive.

INTERMEDIATE RATE OF FREEZING

Up to 1962, long-term preservation of human erythrocytes at sub-zero temperature has been accomplished by either one of two methods. The first utilized a very rapid rate of cooling coupled with extra-cellular cryoprotective agents e.g. sugars or PVP. It was hoped that blood preserved by this method could be directly transfused without the need for further processing. However, sugars were soon excluded because being of low molecular weight, its administration into the circulation produces a substantial increase in the osmotic pressure of the plasma and hence pulmonary haemorrhage and renal damage (Pert et al. 1964). After the exclusion of sugars, PVP was found to be most efficient in protecting the red cells against rapid freezing, but this time, it was its high molecular weight that caused the problems involving its use. Because of its large molecular size, a high percentage of the amount transfused could not be excreted by the kidneys and was retained by the reticulo-endothelial system for very long times (Altemeier et al. 1954, Pert et al. 1963). Even after the introduction of hydroxyethyl starch (HES), (an extracellular cryoprotective substance of high molecular weight which can be easily digested in the circulation by the enzyme amylase), the final product could not be transfused directly after thawing because of the high residual free haemoglobin content.

The second method of blood preservation by freezing employed a slow rate of cooling associated with a high concentration (40% W/V) intracellular additive, glycerol. However, the removal of such high concentrations of glycerol has proved difficult (Meryman 1968b).

Intensive care, considerable amounts of time and large columes of wash solution are required for washing the glycerol out of the red cells without injuring them.

It was such considerations that led to the development of a new method which employed an intermediate rate of freezing (less than 10^{o}C per sec) together with a mixture of low concentration of glycerol and small amounts of sugars as an extracellular additive.

Pert et al. (1963) were the first to show that a glycerol concentration of the order of (14g%) and sucrose (2.76g%) combined with freezing in liquid nitrogen provided excellent protection to the preserved human red blood cells. They also demonstrated the presence of a relationship between the glycerol concentration and the cooling rates (Pert et al. 1965). The range of glycerol concentration that produced the highest red cell recovery was from 14-20 vol.%. Below 14 vol.% glycerol, a high degree of accuracy is required in order to control both the cooling rate and the glycerol concentration. Glycerol concentrations above 40 volume % permitted the use of any freezing rate with good red cell recovery. At glycerol concentration of 20 vol.% a wide selection of freezing rates could be employed.

In the recommended dose (14g%), glycerol was promptly and efficiently removed from the red cells by the so called "batch washing". This type of washing consists of a sequential series of centrifugal sedimentations decantations and resuspensions in a new solution. Because glycerol is used in low concentration, gradient washing are not required. The glycerolized thawed red cells are first diluted with an equal volume, of 30g% sucrose followed by 1000 ml of 0.85 per cent sodium chloride. The erythrocytes are then separated by

centrifugation and washed twice with sufficient volume of isotonic saline. The first wash, a hypertonic sucrose solution, serves as an osmotic gradient to remove the water and the glycerol from the red cells. Utilizing Teflon bags and this method of freezing and washing the authors reported an in vitro recovery of 96 to 99.5% and 24-hour chromium survival in the range of 86 to 95%. The total volume of wash solution used was 2.5 litres and it took about 2½ hours to process one unit. After completion of the wash process the glycerol concentration was reduced from 14g% to 0.1g%.

In 1969 Pert et al. used plastic containers (Teflon and Hemoflex bags) for freezing in liquid nitrogen and were able to adapt the method of continuous-flow centrifugation employing the ADL-bowl for washing red blood cells frozen by their method and obtained almost the same results.

In 1964 and 1965 Krijnen et al. introduced further improvements on the original method proposed by Pert and associates. They showed that 17.5 per cent W/V glycerol is optimal for protection of red cells frozen in flat stainless steel container. The glycerolizing solution recommended by them contained:

glycerol	35% W/V
Sorbitol	4%
Sodium chloride	0.9%

Equal volumes of this solution and packed red cells were mixed in a stainless steel container 255 x 255 x 9 mm and frozen by direct immersion in liquid nitrogen. After thawing the red cells were separated by centrifugation and washed once with 300 ml of 16%

sorbitol in 0.9% saline. This was followed by two successive saline washes. Under the condition of experiment they reported an in vitro recovery of 97 to 98% and autologous in vivo survival with ^{51}Cr of 94 to 99% at 24-hours.

Rowe et al. (1968) have also utilized the low-glycerol-intermediate freezing technique to preserve human red blood cells for transfusion. Equal volumes of packed red cells and glycerolizing solution were frozen in flat stainless steel container (10x10x$\frac{3}{8}$ inch) by immersion into liquid nitrogen. The freeze protective solution contained:

glycerol	28% V/V
Mannitol	3%
Sodium chloride	0.9%

After thawing, the blood-additive mixture was transferred to a plastic bag and washed with a serial centrifugation technique. The first wash consisted of 16% mannitol in isotonic saline, this was followed by two isotonic saline washes. The mannitol acted as an osmotic gradient to extract the glycerol from the erythrocytes and the last two washes for the removal of the residual glycerol, mannitol free haemoglobin, cell debris, platelets and leucocytes. The mannitol was preferred only because it was a commercially available licenced product in the U.S.A. whilst sorbitol was not. The total volume of wash solution used was less than one litre, the red cell recovery was more than 90% and the in vivo survival was $96 \pm 1.5\%$. Rowe and his colleagues have also reported their experience in transfusing 350 units of blood, that were frozen and stored in liquid nitrogen for up to 12 months to recipients with various diseases without adverse reactions.

In (1969b) Valeri and Runck used two different methods, serial

and continuous centrifugation, for washing red blood cells that were frozen with Rowe's method in flat stainless steel container. They showed that washing by serial centrifugation required 0.75 litres of wash fluid and was very efficient as evidenced by the level of the supernatant glycerol (250 mg/100 ml). However, the method required almost two hours and a substantial effort from the technician. Nevertheless, the in vitro recovery, supernatant haemoglobin and in vivo survival were more or less similar with both methods. Using the continuous-flow-centrifugation technique in A.D.L. reusable bowl the processing time was 30 minutes, utilizing $2\frac{1}{2}$ litres of wash solution but resulting in almost double the amount of residual glycerol in the supernatant (536 ± 222) mg/100 ml. With either method the intracellular potassium was not affected by the washing process.

Rowe (1971) and Rowe and Lenny (1972) introduced two modifications into their original method. Firstly they substituted a polyethylene bag, (Hemoflex bag) for the stainless steel container in which the glycerolized blood was frozen. Rowe (1971) indicated that the plastic bags are superior to the metal container for they are easier to handle. Furthermore the invitro recovery was significantly higher and the supernatant haemoglobin was lower with plastic bags than with stainless cans. Secondly, the authors used the Elutramatic, an automated blood washing machine that utilizes the continuous-flow centrifugation principle, for processing the blood frozen by their technique. As a result of these changes the processing time was reduced to 50 minutes for two units instead of two hours.

In 1970 Krijnen et al. also replaced their stainless steel container with a seamless, cylindrical, aerosol can, made of aluminium,

in which they successfully froze red blood cells with a glycerol-sorbitol solution. The can was formed of two separate portions, a screw-cap neck and a body, which were assumbled before use by forcing the former part with a rubber ring into the body. They also developed a five-tailed plastic pack for processing.

Jenkins and Blagdon (1971) were concerned about the safety of Krijnen canister because of the possibility of liquid nitrogen leakage through the joint between the neck and the body. This carries the potential danger of explosion or bacterial contamination. They developed a new method of sealing the same can but it was sealed at the top by a rubber stopper which had a turnover skirt and was wired into place with three turns of copper wire. It was also found desirable to increase the glycerol concentration to 20% W/V. Glycerolized blood was cooled in liquid nitrogen at an average rate of $0.7^{\circ}C$ per second, then stored in the vapour phase of liquid nitrogen at $-150^{\circ}C$. After thawing the blood was transferred to a five-tailed plastic pack, modified to give access to standard U.K. giving ports, and washed with serial centrifugation according to the method of Krijnen et al. (1964). The total red cell haemolysis of blood processed by this method was approximately 10 per cent, and the 24-hour post-transfusion survival, measured by the automated differential agglutination, was from 90-95% (Blagdon 1972). After processing the blood was transfused as packed red cells, with a haematocrit of 75%, in saline. The maximum period of post-thaw storage was 12 hours.

In 1972 Akerblom and Högman described a method of freezing red blood cells that permitted deglycerolization by any of three methods: serial centrifugation (batch washing), continuous-flow centrifugation

and reversible agglomeration. In their method, 100 ml of freeze-protective solution was added to hard packed red cells from one unit and the mixture was transferred into a plastic bag made of Kapton/ Teflon -FEP laminate.

The glycerolizing solution contained

glycerol	55g
Sodium chloride	0.6g
Fructose	1.5g
Glucose	0.25g
Distilled water ad 100 ml.	

The bag was then placed in an aluminium envelope and frozen in liquid nitrogen. After thawing, the blood could be processed by serial centrifugation "batch washing" by transferring it into either a glass bottle or into a five-tailed plastic pack where a hypertonic (3.7%) NaCl solution was added to it in the ratio of 1:2. The red cells were then separated and given three washes with isotonic saline (Akerblom and Högman 1974). Alternatively the thawed blood could be washed in an automatic continuous-flow washing machine (Elutramatic Ultra Flo.) in which two units could be processed simultaneously. If the demand for blood was high, deglycerolization could be accomplished rapidly by the agglomeration washing procedure, the low extracellular sodium chloride helped to fulfill the requirements for this method. The authors (1974) pointed out that it was the actual urgency for the blood that determined the method of deglycerolization. Thus if only a few units are required for a non-urgent case, then the centrifugation methods, either serial or continuous, would be satisfactory because either method yielded a high red cell recovery and low supernatant haemoglobin. When the blood was required in large amounts, then the

agglomeration method would be the most adequate in spite of the higher residual free haemoglobin and the lower red cell recovery.

Following experience in Europe with the low glycerol-liquid nitrogen method it became apparent that this method offered several advantages over the high glycerol-slow freeze technique. The former method provided a wide selection of post-thaw washing processes. Of special importance was the "batch washing"; a method which offered the advantage of high red cell recovery, small volumes of wash solution and the use of equipment available in any blood transfusion service. Possibly the reasons for Blood Banks in America retaining the high glycerol-slow freezing method at -80°C was their inability to obtain a licence for using aluminium containers and the high cost of liquid nitrogen. Furthermore by the time the low-glycerol-liquid nitrogen method has fully developed, a hugh amount of money was already invested in mechanical refrigerators that work at -80°C. However, most of our recent knowledge about the frozen blood came from experience with the high glycerol-slow-freezing technique at -80°C. Little has been published concerning detailed evaluation and experience with blood frozen with low glycerol in liquid nitrogen.

CLINICAL EVALUATION OF THE FROZEN BLOOD

The necessity for quantitative evaluation of the blood recovered after freezing and thawing has been emphasized by many investigators (Rinfret 1963, Tullis 1963). It is not sufficient that a certain method of freeze-preservation yields a high percentage of morphologically intact red cells: of utmost importance is that these cells show both in vitro stability and in vivo viability and function.

It has long been realized that red blood cells frozen in the presence of intracellular cryoprotective agents will, after thawing, become osmotically unstable and, unless the additive is removed, it will undergo excessive haemolysis if resuspended in physiological medium. However, the prolonged experience with the frozen blood showed that even after washing out the intracellular additive, instability in physiological media was a constant feature of all previously frozen-thawed erythrocytes on storage, whatever the method used for its preservation (Tullis et al. 1958, Doebbler and Rinfret 1959). This instability was evident by the continuous increase in the level of the supernatant haemoglobin. This implied that determination of the post-thaw stability in physiological media as well as standardization of physiologically acceptable levels of free haemoglobin in the supernatant fluid must be made before recommending a particular method for clinical application.

Some of the recovered cells might be irreversibly damaged so that they are rapidly removed from the circulation once they are transfused. If the removal of these cells is not accompanied by haemoglobinaemia, then the determining factor of acceptability, as far

as the free haemoglobin is concerned, will be the level of the haemoglobin in the supernatant fluid. Following administration the free haemoglobin will be bound by the haemoglobin-binding protein (hapotoglobin) in the recipient circulation until the latter is saturated. The excess free haemoglobin will remain as such in the blood and, if it exceeds the renal tubular threshold, it appears in urine (Valeri 1965b). Although haemoglobinuria might be well tolerated by healthy as well as patients with normal blood pressure and blood volume, it might, in seriously ill or shocked patients, precipitate acute renal failure (Valeri 1970). However, high levels of supernatant haemoglobin are not uniformally toxic, for free haemoglobin and stromal protein introduced in compatible transfusion are easily tolerated without untowards side effect and has to be separated from the serious effects of incompatible transfusion (Zwilling 1958).

Different procedures undertaken in the preparation of freeze-preserved red cells for transfusion may result in significant physical and biochemical changes of these cells. Thus in spite of the fact that the total concentration of the intracellular cations appeared unchanged, differential cation estimation showed a substantial increase in the sodium and significant decrease in the potassium content of the cells (Doebbler and Rinfret 1965).

ORGANIC PHOSPHATE COMPOUNDS

In the last few years, much attention has been paid to the functional status of preserved red cells, particularly, the ability to deliver sufficient amounts of oxygen to the tissues. In vivo viability is no longer considered the only criterion for successful preservation, as red cells may have normal in vivo survival but are still unable to bind and release oxygen physiologically (Akerblom et al. 1968, Valeri and Hirsch 1969, Fortier et al. 1969, Bunn et al. 1969).

That erythrocyte function might be affected by routine storage was first observed by Valtis and Kennedy in 1954. Those authors noticed that blood stored in acid-citrate-dextrose (ACD) at 4°C for longer than one week had an increased affinity for oxygen, demonstrated by a significant shift to the left of the oxygen dissociation curves. This means that the amount of oxygen unloaded to the tissues by this blood would be substantially lower than that released from fresh blood. Administration of such blood in large volume to anaemic patients would result in shift to the left of the oxygen dissociation curve of the recipients' blood, the extent and the duration of that shift was proportional to the quantity and period of storage of the transfused blood. However they demonstrated that in vivo restoration of the oxygen dissociation curve occurred, but that several hours were required after transfusion in order that the oxygen function would return, even partially, to normal levels.

Other investigators (Bartlett and Barnet 1960) had demonstrated that during storage of ACD-blood at 4°C the organic phosphate compounds of the red cells, especially 2,3 DPG and ATP, progressively decreased.

They estimated that, approximately all the red cell content of 2,3 DPG disappeared in two weeks, however the rate of disappearance of ATP, during this period, was much slower, but increases rapidly thereafter.

Human red blood cells are known to contain large amounts of organic phosphate compounds as a result of metabolic glycolysis. A major proportion of these compounds in a normal red cell is converted to 2,3 DPG and ATP; the former being four times greater in concentration than the second; (Chanutin and Curnish 1967) and in molar concentration it is equivalent to the haemoglobin tetramer (Bunn and Jandl 1970). With the exception of red cells, all the tissues metabolize 1,3 DPG into 3-PG with production of one mole of ATP. In the red cell, however, most of the 1,3 DPG is metabolized through a different route by being transformed into 2,3 DPG first and after completion of the cycle into 3-PG. No high energy phosphate bond is liberated (Bunn and Jandl 1970).

The relationship between the oxygen affinity of the haemoglobin and the level of the red-cell organic phosphates compounds has been demonstrated by several investigators by a great variety of in vivo and in vitro experiments.

Electrophoretic analysis of haemoglobin solutions prepared from fresh blood revealed the presence of a separate zone which moved slower than the haemoglobin itself and this was designated as the B-component (Sugita and Chanutin 1963). This slow moving zone was absent on electrophoresis of haemolysates made up from stored erythrocytes, but it reappeared on addition of either 2,3 DPG or ATP.

Studies of the oxygenation curves of haemoglobin solutions which

have been stripped of organic phosphates showed a very high affinity for oxygen evidenced by a substantial shift to the left of the oxygen dissociation curve (Benesch and Benesch 1967). Addition of increasing amounts of 2,3 DPG or ATP resulted in a progressive reduction of the oxygen affinity and restoration of the oxyhaemoglobin dissociation curves to normal (Benesch and Benesch 1967, Chanutin and Curnish 1967).

When inosine was added to stored blood, it resulted in restoration of the deficient 2,3 DPG and ATP compounds of the red cells (Bartlett and Shafer 1961). Moreover, Akerblom et al. (1968) demonstrated that addition of inosine to stored blood reduced the oxygen affinity of the haemoglobin to normal and shifted to the right the oxygen dissociation curves.

From all these observations, there remains little doubt that the increased oxygen affinity observed by Valtis and Kennedy is primarily due to the deficiency of 2,3 DPG and ATP (Benesch and Benesch 1967, Chanutin and Curnish 1967). It has been shown that in molar concentrations ATP is as effective as 2,3 DPG in lowering oxygen affinity (Benesch and Benesch 1967). However, since 2,3 DPG exists in unusually high concentrations, four times greater than ATP, (Chanutin and Curnish 1967) and since ATP concentration is but slightly altered, during the first two weeks of storage in ACD at $4^{o}C$ it can be argued that the concentration of 2,3 DPG correlates very well with oxygen affinity of stored blood alone (Bunn et al. 1969). Benesch et al. 1968 maintained that 2,3 DPG reacts mole formole with deoxy-haemoglobin and they determined an equilibrium constant of this reaction. Because of this binding 2,3 DPG lowers the haemoglobin affinity for

oxygen. When this equilibrium constant was applied to the high haemoglobin concentration that exists in the erythrocytes, it was found that a significant increase in oxygen affinity would occur only when the cellular 2,3 DPG concentration decreased to half the normal values (Bunn et al. 1969).

The clinical significance of the increased affinity of haemoglobin to oxygen is not clear. Valtis and Kennedy 1954 observed that the administration of erythrocytes that have increased affinity for oxygen resulted in a shift to the left of the oxy - haemoglobin dissociation curve of the recipient blood, and that the extent and duration of this shift is related to the volume and period of storage of the transfused blood as well as the general condition of the recipient (O'Brien and Watkins 1960). Thus anaemic patients, who received large quantities of stored blood may suffer from a shortage of oxygen supply to their tissues for several hours until the transfused cells resynthesize the necessary amounts of 2,3 DPG and ATP. However Valeri (1971) reported that transfusion of stable nonsurgical anaemic patients with 3 to 5 units of washed blood that has been stored at $4^{\circ}C$ for two to three weeks had no effect on the systemic oxygen consumption or cardiac output. He also found that the systemic arterio-venous difference in oxygen content was greatly reduced soon after transfusion, but within four hours it had returned to normal together with partial restoration of the intracellular 2,3 DPG content and an elevation in the p50 (Partial oxygen pressure at which half the haemoglobin is oxygenated) value of the oxygen dissociation curve. Nevertheless he emphasized that in certain clinical situations in which large amounts of blood are needed, it

may be necessary to transfuse red blood cells which are able to circulate and function immediately upon infusion.

Transfused red blood cells that have been completely depleted of its 2,3 DPG, start to build up this compound immediately after infusion. Using the differential agglutination technique of Ashby to distinguish the donor cells which had been preserved in ACD, Valeri and Hirsch (1969) found that at least 25% of the final level of 2,3 DPG was restored within three hours after transfusion and by 24-hours more than 50% was resynthesized. Thereafter the rate of synthesis is slow and reach a plateau in nine to 11 days. Contrary to 2,3 DPG, the ATP content of the transfused cells increases rapidly during the first 24-hours and this was combined with rapid reduction in the sodium ions (Fortier et al. 1969). Potassium ion content of the donor red cells increases very slowly after transfusion and this was apparently related to the absolute level of 2,3 DPG (Valeri and Hirsch 1969). Other investigators (Beutler and Wood 1969) reported rapid rate of restoration of 2,3 DPG of the transfused cells. Approximately 50% of the 2,3 DPG that disappeared on storage was restored within four hours of transfusion.

The pH of the cell is an important factor in determining the level of red cell 2,3 DPG (Bunn and Jandl 1970). Rörth (1970) also confirmed that the red cell 2,3 DPG concentration was dependent on the intracellular pH and indicated that this dependency reflected the effect of pH on the red-cell enzymes 2,3 DPG mutase and 2,3 DPG phosphatase. A decrease in the red-cell pH resulted in a reduction of the 2,3 DPG content and thereby an alteration in oxygen dissociation curve due to increased oxygen affinity of the haemoglobin. Blood

preserved in solutions with reasonably high pH maintains its 2,3 DPG concentration, and hence its haemoglobin function, at nearly normal levels for approximately two weeks (Dawson et al. 1972). Unfortunately such high pH solutions have a deteriorating effect on the ATP content and hence on red cell viability (Dawson et al. 1972).

In a search for an optimal pH that maintains the levels of both 2,3 DPG and ATP for long periods Dawson et al. (1972) found that a preservative with pH 6.0 was most suitable, as it conserved the 2,3 DPG similar to these with higher pH and in addition it had an intermediate effect between high and low pH solutions in maintaining the ATP concentration. For this reason CPD and CPD adenine are considered superior anticoagulants than ACD and ACD adenine for preserving the blood at $4^{o}C$ (Chanutin 1967). Blood preserved with CPD showed a slower rate of disappearance of its 2,3 DPG and ATP content.

Akerblom et al. (1968) demonstrated that the increased oxygen affinity of haemoglobin which occurs during storage of the blood in ACD or ACD-adenine could be reversed by the addition of inosine. They pointed out the potential advantage of late addition of inosine (after 16 and 35 days of storage) when the glycolytic processes are inhibited by the loss of ATP and the decreased pH. ATP is known to affect the rate of glycolysis by acting as cofactor for the hexokinase reaction (Strumia et al. 1970). When inosine is added to stored blood it functions as a substitute for glycolysis by being transformed to ribose-phosphate compounds, the latter could be easily converted to glyceraldehyde-3-phosphate thereby, bypassing the hexokinase reaction. Bunn et al. (1969) added inosine to fresh blood at the time of collection and in so doing were able to maintain the oxygen

affinity for haemoglobin at the initial level for about two weeks. Strumia et al. 1970 confirmed the early observations that the addition of adenine and inosine to stored blood increased the ATP content and thus improved the viability of the erythrocytes and prolonged the storage period to 42 days with post-transfusion survival greater than 70% after 24-hours.

The transformation of glyceraldehyde-3-phosphate (G-3-P), resulting from ribose and which in turn is derived from splitting of inosine, into 1,3 DPG requires the presence of glyceraldehyde-3-phosphate dehydrogenase (G-3-PD) and the availability of coenzyme I, nicotinamide adenine dinucleotide, (NAD) as hydrogen carrier. However, the accumulation of NADH was found to have an inhibitory effect on the enzyme (G-3-PD). Addition of pyruvate would ensure the continued availability of NAD, while the pyruvate itself is converted to lactic acid. Pyruvate and inosine and inorganic phosphate all in a final concentration of 10 mM were successfully used, in vitro to resynthesize the lost 2,3 DPG in blood stored for 21 and 28 days (Oski et al. 1971).

While inorganic phosphates and pyruvates represent no problem as far as toxicity is concerned, the intravenous administration if inosine, especially in the amounts required for the resynthesis of ATP and 2,3 DPG in multiple units, may have an adverse effect on the recipients. Degradation of inosine results in the production of uric acid and hypoxanthine which might persist in the circulation for 24-hours (Valeri 1971). Again adenine is metabolized to 2,8-dioxyadenine which is potentially nephrotoxic (Shields et al. 1970). Strumia and Strumia (1972) suggested a single washing of the red cells

prior to transfusion to remove these potentially toxic metabolites.

Maintenance of the 2,3 DPG and ATP levels in stored blood up to the time of transfusion could now be achieved by any of the different methods of freeze-preservation (Valeri 1970, 1971). Many investigators reported that the process of freezing, thawing and washing of the red cells had only slight or no effect on the concentration of the 2,3 DPG and ATP content of the frozen erythrocytes (Akerblom and Kreuger 1974, Valeri (1974a). Naturally the amount of these two compounds, recovered with red cells after thawing and washing would depend on the concentration present prior to freezing which in turn is affected by the period of storage in the liquid state as well as the anticoagulant used for collection. In this respect, CPD had again shown superiority over ACD anticoagulant (Valeri (1974a). Red blood cells collected on either ACD or CPD and stored at $4^{\circ}C$ for up to seven days before freezing maintained a satisfactory concentration of 2,3 DPG and ATP after storage at $-80^{\circ}C$ for about 30 months.

O'Brien and Watkins(1960) have reported that if the blood is frozen within five hours of donation (in the presence of high concentration of glycerol and utilizing the slow freeze-thaw technique) it maintained a normal oxygen transport function after thawing, washing and resuspension, irrespective of the anticoagulant used.

However, because it might be inconvenient to freeze all the blood immediately upon collection, combination of both liquid and freeze preservation could be used to obtain maximum benefit from each method and at the same time to overcome the ever existing problems of supply and demand. Blood was collected in the normal way in any of the routine anticoagulants, stored at $4^{\circ}C$ and if it is not used until it

is outdated, or depleted of the vital metabolic compounds, it could be frozen after rejuvenation with high concentrations of organic phosphate regenerating compounds (Valeri and Zaroulis 1972). The latter consists of pyruvate, inosine, glucose and phosphate with or without adenine. Because post-thaw washing is a necessary step with all method of freezing in current use today there should be no fear of the potential hazards of these compounds on transfusion. Valeri and Zaroulis (1972, 1974b) applied this technique for both ACD and CPD outdated blood which has been frozen with either the high glycerol slow-freeze-thaw technique or with the low glycerol rapid freeze-thaw technique and obtained satisfactory post-transfusion in vivo survival and oxygen transport function.

Normal red cell oxygen-transport function depends, mainly on the concentration of 2,3 DPG inside the cell (Benesch and Benesch 1967, Chanutin and Curnish 1967, Valeri and Zaroulis 1972, Valeri 1974a and 1974b). The normal value of this compound is about $12 \pm 1.2\mu$ mole per gram haemoglobin (Valeri and Hirsch 1969, Valeri and Zaroulis 1972). Normal values of ATP content of the red cells were also reported by Valeri and associates in 1969 and 1972, to be $3.9 \pm 1.0\mu$ moles per gram haemoglobin. Dern et al 1967 observed a correlation between the intraerythrocytic ATP concentration and the post-transfusion in vivo viability of stored red blood cells. This work was further extended by Strumia et al 1970 and Strumia and Strumia 1972 who suggested that 70 per cent of the original ATP content of the red cells at the day of collection, was compatible with a 70% post-transfusion survival at 24-hours.

POST TRANSFUSION SURVIVAL

Introduction

The term survival indicates the percentage of transfused red cells that remain in the recipient circulation in a physiologically active form after transfusion (Turner 1969, 1970).

Although in vivo survival is the ultimate and most decisive criterion of efficacy of a proposed technique for red cell preservation, it is relevant only after satisfactory in vitro evaluation tests. It must be emphasized that there is no positive correlation between the in vitro recovery and in vivo survival percentage, for in many instances in vitro red cell recovery may be very poor yet the recovered cells survive very well following transfusion (Mollison et al. 1952); the reverse is also true (Strumia et al. 1958b, Strumia et al. 1960b).

Freezing and thawing bring about certain degrees of damage to the red cells, some of the cells are irreversibly destroyed by the process of freezing, thawing and post thaw processing, and these are rapidly removed from the recipient circulation after transfusion (Strumia et al 1960b). Other cells are reversibly affected by these processes, as evidenced by the increase in intracellular sodium and decrease in potassium ion concentrations, but upon transfusion they are rapidly restored to normal (Crawford and Mollison 1955, Valeri and Hirsch 1969) and thus resume the potential for long-term-survival (Valeri 1971). Since the discovery of red cell preservation by freezing, a great number of methods have been proposed for the determination of the percentage of the red cells that survive in the recipient circulation after transfusion. However, at the present time there is no in vitro

determination that can predict the number of cells capable of surviving in the recipient circulation after transfusion. The only way of determining the number of irreversibly damaged cells is by in vivo measurements of post-transfusion survival of red cells.

Many investigators have shown that red cells which are irreversibly damaged by freezing are removed from the circulation within the first 24 hours post-transfusion and those cells which survive this period have the potential for surviving normally after that (Strumia et al 1960b, Schmidt and Steinfeld 1960, Mollison 1972, Szymanski and Valeri 1968, Valeri and Runck 1969a, Valeri 1971). However in vivo, long term survival of the preserved red cells is largely determined, not by the technique of preservation or by the duration of storage in the frozen state, but by the recipient's general condition and intravascular environment, for the recipient in order to be able to restore the irreversibly injured cells, his circulation must be free of any immunologic, toxic or chemical factors (Szymanski and Valeri 1971).

In the past red cell survival was determined by the so-called differential-agglutination technique (First proposed by Ashby in 1919). This involved the transfusion of test cells which are antigenically different, but compatible, from that of the recipient. The percentage of test cells surviving at a certain time could be determined in a sample of blood by separation of the two populations of cells using a highly potent serum to agglutinate the recipient cells only. Nevertheless, the method was abandoned because of the technical difficulties. However, in 1968 Szymanski and her associates automated the original differential agglutination technique using the Technicon Auto Analyzer. They claimed that this made possible the study of the

effect of different methods of preservation on the in vivo survival in the same recipient.

Because some of the transfused red cells are removed very rapidly, within the first few minutes of transfusion, an accurate estimation of the in vivo survival can only be made if the red cell volume of the recipient is determined (Mollison 1972). Study of the survival of preserved red cells involves comparison of the ratio of transfused cells to those of the recipient. The survival is computed from the alteration of this ratio from the time of administration. When a great proportion of the transfused cells disappear rapidly from the recipient's circulation before mixing is complete, the initial value of comparison is diminished, this results in an overestimation of the red cell volume and hence a falsely high in vivo survival. For this reason precise estimation of the red cell volume is of the utmost importance. In 1958, Mollison, Robinson and Hunter introduced the use of two different isotopes, one for the test cells and the other for the control cells, to estimate the recipient red cell volume. They suggested chromium ^{51}Cr for labelling the preserved, test cells, and phosphorus 32p to label the recipient freshly drawn red cells, the control. However as 32p is β -emitter it presents some difficulty in radioactive counting (Dacie and Lewis 1970 and Button et al 1965).

In 1962 Doebbler et al introduced the double-chromium technique. In this method the same radioactive isotope, ^{51}Cr, was used to label both the test and the control cells. Fresh recipient red cells were first injected after labelling with the minimal possible dose of radioactive chromium. After allowing sufficient time for mixing, peripheral blood samples are withdrawn to supply an estimate of red

cell volume. The test cells, labelled with 10 times the radioactive dose, are then injected. Survival at any required time is computed by correction for the chromium count observed at this time assuming normal survival of the control cells. Because very small dose of radioactive label is used with the control cells any small variation in its survival is further minimized and thus does not affect the results. However, a major disadvantage of this method is the requirement of two procedures for every survival determination (Button et al. 1965). Moreover, the process lacks complete accuracy because of the use of two samples for injection and two standards for counting (Mollison 1972).

Red cell volume can be determined in healthy subjects indirectly, from an estimate of plasma volume and peripheral venous haematocrit and a correction factor. The latter corrects the directly measured peripheral venous haematocrit to the total body haematocrit. When total body haematocrit (TBH), calculated from direct independent measurement of red cell volume and plasma volume, is compared with the peripheral venous haematocrit (PVH), the PCV after correction for the trapped plasma, the former is usually found to be smaller than the latter, the ratio TBH/PVH is close to 0.9 (Glass 1973).

Plasma volume may be determined by the use of Evans Blue dye (Gibson & Evans 1937) and the dilution technique. However the use of this method was abandoned for a more accurate method using radioactive iodine-labelled albumin (Button et al. 1965). At first iodine (^{131}I) was suggested for this purpose, but the difficulty in separating the superimposed peaks of ^{131}I and ^{51}Cr led to its abolishment (Button et al. 1965). The best radioactive iodine suitable for

labelling the human serum albumin is ^{125}I, it has a half-life of 59 days and this permits extended storage. Moreover because it disintegrates by pure gamma emission it can easily be separated from that of ^{51}Cr (Button et al. 1965). The most popular protein used for determination of plasma volume is human serum albumin. A bigger molecular weight protein might give a better estimate of the plasma volume, but albumin offers the advantage of being heat-treated to inactivate serum hepatitis virus (Glass 1973).

Although iodinated serum albumin offers a satisfactory estimate of plasma volume in healthy subjects its use in the diseased recipient is not reliable, due to the rapid loss of the albumin molecules (while mixing is still incomplete), into the extravascular spaces as a result of increased capillary permeability in these conditions (Valeri et al 1973). For this reason, it is always advisable not to compute the blood volume in diseased patients from estimations of the plasma volume alone. Independent estimations of the red cell volume (RCV) and plasma volume (PV), by using respectively radioactive ^{51}Cr and ^{125}I labelled serum albumin, is the most reliable method under these conditions (Glass 1973 - Valeri et al. 1973).

Red cell volume and plasma volume can be estimated from the body surface area of the subject, these are extracted from charts based on the height and weight of the person at the time of transfusion

For men the RCV = BSA (m2) x 1091

For women the RCV = BSA (m2) x 806

and the plasma volume could be calculated as follows

For men PV = BSA m2 x 1561

Valeri et al 1973, Szymanski & Valeri 1971.

As a red-cell label ^{51}Cr, in the form of sodium chromate, is the most suitable (Glass 1973). It was first introduced by Gray and Sterling in 1950 for the estimation of the red cell volume, but Gibson and Scheitlin 1955 were able to adapt it for the determination of post-transfusion survival of stored human red cells. Since then most of the work conducted for study of the in vivo survival of red cells has employed radioactive chromium. Its use in experimental and clinical studies has made possible a better understanding of the in vivo behaviour of red cells preserved under different conditions of storage.

Radioactive chromium (^{51}Cr), has a half-life of 27.8 days (Dacie and Lewis 1970). The label is usually in the form of anionic hexavalent compound and the most commonly used salt is the sodium chromate. When added to red-cell suspensions radioactive chromate (^{51}Cr) rapidly diffuses into the inside of the red cell where it is reduced to the trivalent form. As the red cell membrane is impermeable to the trivalent chromium the chromium released from the damaged red cells would be incapable of labelling others (Gibson & Schietlin 1955). Inside the red cell ^{51}Cr strongly binds with the haemoglobin, specifically, to its β-polypeptide chain (Dacie and Lewis 1970) and it is not toxic in the small doses used. However, doses larger than 5 µg/ml block the enzyme glulathione reductase and if more than 10 µg/ml it interferes with glycolysis probably due to its oxidising effect.

Chromium uptake:

Estimation of the post-transfusion survival of the red blood cells labelled with radioactive chromium is based on the principle that the

red cells pick up the chromium at the time of labelling and will keep it all the time they survive in the circulation until it is released, and hence removed from the circulation after their destruction.

The percentage survival on a certain day is determined by comparing the radioactivity of blood sample withdrawn on that day to that of time zero.

After the addition of chromium the process of labelling is allowed to proceed for about 20 minutes at room temperature, following which the excess unattached chromium is removed by washing the cells twice with a sufficient volume of sterile isotonic saline (Dacie and Lewis 1970). When the chromium is used to label the recipient's own cells for estimation of blood volume it is preferable to expose the cells to as little trauma as possible in order to avoid falsely high results. In this case the excess hexavalent chromium could be reduced to trivalent inactive compound by the addition of ascorbic acid (Doebbler et al 1962). However, Valeri (1968b) showed that even this process is not necessary since the chromium uptake is almost complete whether the cells were fresh, ACD-stored or previously frozen. At the same time he did recommend not to wash the red cells after labelling in order to avoid selective loss in vitro of the irreversibly damaged cells. Differential chromium labelling of stored red cells has also been studied by Valeri (1968b). In his experiments, he separated the red cells of both ACD-stored and previously frozen-thawed blood into three fractions on the basis of cellular density. Preferential labelling with chromium was noted, the lightest fraction showed significantly higher uptake than the heaviest one.

When the survival of the red-cells was estimated by the

differnetial agglutination technique and the radioactive chromium labelling technique a difference between both methods was consistently observed (Emerson and Bove 1960); the decline in chromium radioactivity was greater than could be accounted for by red cell loss. This was attributed to spontaneous leakage of chromium from the intact erythrocytes and was referred to as "Chromium elution". Elution of ^{51}Cr from intact cells differs from one person to the other and it usually occurs in two phases, a rapid and slow phase. The rapid phase of chromium elution occurs within the first 24 hours post-transfusion and was estimated to be about 10% of the chromium label (Dacie and Lewis 1970) and is influenced by the technique of ^{51}Cr labelling (Valeri and Szymanski 1973). The slow phase of elution proceeds at a rate ranging from 0 - 1.06% per day (Valeri and Szymanski 1973) and has a half-time about 77 days (Dacie and Lewis 1970). Because of the substantial variation in the extent of elution, there is no valid correction factor. Even when the observed radioactivity is corrected for elution it does not give rise to survival curves which resemble those obtained with Ashby method, and hence there appears little benefit in applying such corrections (Dacie and Lewis 1970).

Measurement of radioactivity in frozen blood

Estimation of the post transfusion survival of previously frozen blood is based on the observation that chromium uptake by, and elution from the red cells is not influenced by the processes of glycerolization, freezing, thawing and washing (Morrison et al. 1968, Valeri and Runck 1969a). The last named authors demonstrated that red cells stored in the frozen state for up to six years had chromium uptake and elution characteristics similar to those stored for short duration

and that the rate of chromium elution was not accelerated for these cells.

The twenty-four hour post-transfusion survival represents the percentage of the transfused red blood cells that remain in the circulation after that time. All those who have studied the post-transfusion survival of frozen or unfrozen blood have shown that red-cell which survive the first 24 hours after transfusion have normal long-term survival (Mollison 1972). However Dacie and Lewis (1970) suggested that the most significant single measurement in red-cells survival studies is the $T\frac{1}{2}$ ^{51}Cr, a normal value for this measurement lies between 25 and 33 days (Dacie and Lewis 1970). The mean life span of chromium labelled red cells (T-half Value) is usually expressed as the time in days when half the chromium label is still present in the circulation. As far as the frozen cells are concerned, red cell life span is not influenced by the preservation injury, nor is it affected by the duration of storage in the frozen state or the technique of freezing (Szymanski and Valeri 1971). On the other hand life span of the donor cells may be influenced by host factors, e.g. immunological chemical, mechanical or unidentified toxic factors.

Experience and Variables:

The earliest report of measuring the post transfusion survival of previously frozen red cells was that of Sloviter (1951b). He utilized radioactive (^{32}p) and radioactive (^{51}Cr) to tag rabbit red-blood cells which had been previously frozen and thawed in the presence of glycerol. He found that both the control and frozen cells have approximately the same survival values during the first few days.

It was suggested that many factors may have an effect upon red cell survival e.g. the method employed in freezing, period of storage before and after freezing, temperature and duration of storage in the frozen state and the resuspension media.

Thus Brown and Hardin (1953) showed that as the storage temperature decreases the survival percentage increases. Using the differential agglutination technique to distinguish the donor cells and radioactive 32p method to determine the red cell volume of the recipient they studied the survival of red-cells stored at -70°C in glycerol for 6¼ months and that stored at -15°C for 50 days. The former cells showed an in vitro recovery of over 90% and post transfusion survival of 64%, while for the latter the recovery was 79% and the survival was 47%.

Chaplin et al. (1957) used radioactive chromium (^{51}Cr) to label red-cells, that were previously frozen at -20° for 13 months in buffered citrate-glycerol with added ascorbic acid, and that stored at -45°C for 18 months in buffered citrate glycerol. After labelling, the red cells were washed three times in saline and 50 ml of the washed red cell suspension were used for autotransfusion. Plasma volumes were determined using Evans blue dye. The 100% retention value was determined by extrapolation of the radioactivity at 10, 20, and 40 minutes back to zero time. They reported that the red cells stored at -20°C showed a 24 hour post-transfusion survival of 58% of the cells transfused. By contrast, those cells stored at -45°C showed a 24-hour post-transfusion survival of 96% of the transfused cells.

Previously Chaplin et al. (1956) showed that red cells modified

with glycerol in a final concentration 30% W/V and stored for 21 months at -79°C had a recovery of 90% and post transfusion survival of 80% and indicated that the post transfusion survival was not affected by the period of storage. The absence of progressive deterioration suggested that the metabolic processes were arrested at -79°C, this was further supported by the absence of changes in the concentration of dextrose during the period of storage for one year. In this series of experiments they utilized the differential agglutination technique for the estimation of survival and 32p and other methods for determination of the red cell volume. In some of the cases post transfusion survival was determined both by the differential agglutination method and by labelling the red cells with radioactive chromium (^{51}Cr). They indicated that the estimates of survival by the two methods agreed quite well, but the method of labelling with ^{51}Cr was far more convenient than the method of differential agglutination.

Strumia et al. (1958a) studied the post-transfusion survival of five human blood donations frozen and thawed, in the presence of sugars, without an appreciable period of storage. They reported that the 24 hour post-transfusion survival of the previously frozen cells was only 2% below the optimal established for fresh autotransfused erythrocytes. After the first 24 hours the declining curve of survival of the frozen cells was parallel to that of the fresh blood. Under the condition of their experiments the red-cell post-transfusion survival was found to be dependent on the storage temperature. With storage at -58°C both rates of recovery and survival deteriorated rapidly. At -70°C, although the recovery was fairly maintained for about 40 days, survival progressively deteriorated. At -93°C both

recovery and survival were satisfactorily maintained for 6 months.

In 1960b Strumia et al reported the results of further studies on the post transfusion survival of blood frozen in the presence of dextrose and lactose stored for longer periods in the frozen state. They showed that the 24 hour post-transfusion survival of ^{51}Cr-labelled red cells frozen at -93°C were maintained at a steady level of 77% of the original amount of the red cells frozen for at least two years. This compared with an optimal survival of fresh autologous ^{51}Cr-labelled red cells which averaged 94% ± 2 and that of ACD liquid stored blood after 21 days of storage which varied between 70 and 84%. They maintained that the post-transfusion survival of red cells frozen with the addition of sugars did not necessarily follow the recovery in vitro, for in their experiments whole ACD blood frozen in the presence of 15% and 25% of dextrose showed a very high in vitro recovery but upon transfusion most of the red cells were immediately destroyed. They also indicated that the red cell life span was not related to the length of storage in the frozen state and that cells which have not been damaged in the process of freezing and thawing would survive normally while those damaged by freezing and thawing were immediately removed upon infusion.

That storage at a temperature of -80 or -120°C for very long periods was practical and that the survival of red cells was independent of the duration of storage has been amply demonstrated. Tullis et al (1958) studied the post-transfusion survival of 94 units of blood which were glycerolized, frozen and stored for up to 19 months at either -80 or -120°C. Post-transfusion survival was normal and appeared therapeutically equivalent to cells stored in ACD at 4°C for up to

21 days. By 1960 (Tullis et al 1962) more than 1300 units of glycerolized frozen blood stored for about 44 months had been transfused with normal post-transfusion survival, erythrocyte behaviour and clinical response. Valeri and Runck (1969a) reported that human red blood cells preserved with 45% W/V glycerol, frozen by slow freeze thaw technique and stored at -80°C for up to six years and then deglycerolized by continuous centrifugation with electrolyte solutions, stored for 24 hours at 4°C showed acceptable clinical post-transfusion survivals. They also claimed that 24-hour post-transfusion survival was significantly related to the intracellular potassium content. By contrast to this, Huggins processed red blood cells that were stored for more than two years and showed that they had decreased post-thaw stability while cells stored for more than three years washed by his technique and kept at 4°C for more than 12 hours had a post-transfusion survival of less than 60%. Valeri and Runck (1969b) evaluated the method of freezing which employs the low glycerol liquid nitrogen refrigeration and stainless steel container by measurement of the post-transfusion survival of chromium labelled autologous red cells. They reported that the in vitro recovery was more than 90% and the 24-hour post-transfusion survival was approximately 85%. They also found that the post-trnasfusion survival was inversely proportional to the level of supernatant haemoglobin and directly proportional to the intracellular potassium level at the time of transfusion.

Szymanski and Valeri (1971), using the automated differential agglutination technique, studied the 24-hour red blood cell curvival and the life-span of the red cells in stable anaemic patients to explore the relationship between the red cell life-span and the method

of preservation. They came to the conclusion that the red cell life-span was not influenced by either the duration of storage at +4 or -80°C or the method of preservation but it might be affected by the in vivo environmental host factors e.g. immunological, chemical, mechanical or other unidentified toxic factors.

Another problem that is related to survival of frozen red cells is the type of resuspension medium used for reconstitution of the thawed-washed erythrocytes and period of storage at 4°C post-thaw. Schmidt and Steinfeld (1960) approached this problem in their studies of the post-thaw stability of red cells that had been previously frozen and stored at -45°C for 3 months. Evaluation of the recommended resuspension media was performed by testing the post-transfusion survival of the cells stored, after thawing, in that medium for the required period. After labelling with radioactive ^{51}Cr, the preserved cells were washed three times with saline and resuspended in saline to a haematocrit of 40%. Blood volume of the recipient was determined either by Evans blue solution or by ^{131}I-iodinated serum albumin. They found that although resuspension media, had little effect on in vitro recovery, it substantially affected in vivo survival. Red cells previously frozen and stored at -45°C for three months could be further stored for an additional ten days at ordinary refrigerator temperature. With reconstitution in the proper medium, the in vivo survival was over 85% after both auto - and homotransfusion. Valeri (1965a) also studied the effect of resuspension media on the post-thaw stability and the post-transfusion survival. Red-blood-cells were frozen and stored at -80°C for one month, then deglycerolized labelled with radioactive chromium and resuspended in one of three

media: autologous plasma, an artificial 5% human albumin and outdated 5% albumin medium. The 24-hour post-transfusion survival was satisfactory (70% or more) after a post-thaw storage period of 21, 12, and 6 days respectively.

RESUSPENSION MEDIA

Since the early days of experimental work on frozen blood, investigators resuspended the thawed-washed cells in a physiological medium. Thus Sloviter (1951a) reconstituted the previously frozen-thawed-washed erythrocytes in ACD plasma. However once processed the reconstituted blood had to be used immediately, without further storage at 4°C, because of the potential hazards of possible bacterial contamination. With the introduction of the Cohn fractionator, a closed semiautomated system, for both glycerolization and processing (Tullis 1956) it seemed probable that sterility could be maintained and thus storage of the blood after processing at 4°C was attempted. The problem that emerged was one of finding a medium that maintained the stability of the processed cells up to the time of transfusion. Tullis et al (1958) reported their experience with 94 units of blood that have been glycerolized, stored frozen at -80°C or -120°C for up to 19 months, deglycerolized, reconstituted and stored at 4°C for up to 11 days and then used clinically. Following deglycerolization the packed cells were resuspended in 60 ml of a medium that contained:

400 mg % dextrose
20 m Eq/L of potassium
2 g % (final concentration) albumin

The pH of the final product was 7.1. The authors indicated that storage at 4°C permitted transportation and the use of the blood in locations far away from the processing centres. However, because of the progressive accumulation of free haemoglobin in the supernate, the product was used only within one week of thawing. On the day of processing the supernatant haemoglobin was 150 mg/100 ml but after one week of storage at +4°C it increased to 240 mg/100 ml. The reasons for such lysis was not known and it was found later that it is a common feature of all frozen-thawed erythrocytes after any of the processing methods (Doebbler and Rinfret 1959). This implied that tests for post-thaw stability are as important as any other in vitro tests used for evaluation of a proposed freezing method. Substitution of 30 ml of autologous ACD plasma for the dextrose-potassium-albumin media had greatly reduced the haemoglobin level in the supernate (Tullis et al. 1958). However, because of the scarcity of the plasma, (it was fractionated into its valuable components) and because of the potential danger of transmission of serum hepatitis, other resuspension media had been sought by the group of investigators at the U.S. Naval Hospital in Chelsea, Mass. (Haynes et al. 1960). A solution of 5% serum albumin was found to be satisfactory for this purpose. Previously frozen packed red cells were resuspended to a volume of 500 ml with 5% albumin solution and used successfully as a volume expander and for volume replacement in surgical patients. Up to 1960 a total of 1014 units of blood were transfused in serum albumin, in addition 154 units have been given in their own plasma. As regards the therapeutic efficacy there was no clinical difference between 5% albumin solution and the plasma as a resuspension media.

After the transfusion of 500 units of frozen red blood cells resuspended in heated albumin solution, not a single case of serum hepatitis was detected. On the other hand, of the 154 units, of frozen blood transfused after resuspension in their autologous plasma, one patient developed the disease after receiving two units of such a product. In addition to saving plasma for fractionation and minimizing the incidence of serum hepatitis, resuspension of frozen red blood cells in 5% albumin solution made it possible to "tailor" the final product according to the patient's need (Haynes et al. 1962). These last authors indicated that blood which was previously frozen and reconstituted in 5% albumin solution could be stored in a conventional refrigerator at +4°C for up to 14 days, during this period the blood could be used for medical and surgical patients as well as priming the pump-oxygenator in cardiac surgery. Up to the first of September 1961, Haynes et al used 2250 units of frozen washed blood for the transfusion of 653 patients, most of these units were resuspended in 5% albumin medium which contained also NaCl, potassium phosphate and glucose. They observed far less incidence of febrile reactions, caridovascular pressor effects and urticarial reactions than with the standard ACD - liquid stored blood. Furthermore the incidence of serum hepatitis was believed to be diminished with the use of previously frozen red blood cells resuspended in albumin medium. In spite of the fact that such blood was completely devoid of platelets and blood clotting factors, the bleeding problems which were commonly reported with the use of massive transfusion of ACD- liquid stored blood were not observed.

Schmidt and Steinfeld (1960) examined 40 resuspension media to

choose the most suitable medium for preserving the cellular integrity. Their criteria for suitability were in vitro haemolysis and cellular fragility. The media recommended by them for resuspension of red-blood cells that were previously frozen and thawed contained:

Na Citrate	5.82 g
Citric acid	1.21 g
Dextrose	3.0 g
Na Cl	5.56 g
$Mg\ Cl_2$	0.2 g
K Cl	0.374 g
$Na_2\ HPO4$	0.268 g
$Na_2\ SO4$	0.710 g
Water to	1000

using this media allowed red blood cells that were frozen and stored at $-45^{\circ}C$ for three months to be kept for an additional ten days at standard refrigeration temperatures. Storage for ten days in the above described media at $+5^{\circ}C$ causes no more than 3 per cent in vitro haemolysis and after transportation the red blood cells had in vivo survival of over 85% following both auto and homo transfusion. The authors indicated that the advantage of their medium was that it did not contain any protein and therefore was easily autoclaved and stored.

Murray et al. (1962) used what they called "synthetic plasma" which was formed of:

Serum albumin	5%
Na Cl	0.15 M
Dextrose	0.4%
Potassium phosphate monobasic	0.0025 M

Using the "synthetic plasma" the authors were able to keep the blood,

after thawing and processing, for 21 days in a similar fashion to ordinary ACD blood. Reconstituted blood stored for 12 days at 4°C had a 24-hour post-transfusion survival of 77.8%.

Because of the progressive increase of free haemoglobin and potassium in the supernatant fluid on storage at 4°C, washing the red cells was recommended before transfusion to reduce the level of these two substances in the transfused blood (Tullis et al 1958). However, major clinical difficulties, haemoglobinaemia, haemoglobinuria and acute renal insufficiency were noted with the transfusion of deglycerolized albumin resuspended blood that had been stored at 4°C for three days and then washed before transfusion (Valeri (1965a). Because no such difficulties were observed with red blood cells resuspended in plasma it was suspected that these complications were related to the albumin resuspension media itself. Albumin solutions were found to be unstable when diluted to 5% in the presence of dextrose or when it is outdated (Tullis et al 1963). For these reasons, evaluation of resuspension media was undertaken by Valeri (1965a and 1965c). He reported that, with autologous plasma as a resuspension medium, the supernatant haemoglobin in the unit ranged from 200 mg to 1750 mg on the day of deglycerolization and from 350 to 3150 mg on the day of transfusion, after storage at 4°C for one to 14 days. Using 5% albumin medium and storage at 4°C for one to 17 days, the supernatant haemoglobin in the unit ranged from 120 mg to 2365 mg on the day of deglycerolization and 350 mg to 6130 mg on the day of transfusion (Valeri 1965c). He also found that the resuspension media affect the post-transfusion survival of the frozen red cells that were stored at 4°C after washing. Deglycerolized red cells resuspended in autologous plasma and stored for 21 days at 4°C showed an acceptable, (more than 70%), 24-hour post-transfusion

survival. On the other hand deglycerolized red cells resuspended in 5% albumin had a 24-hour survival less than 70% if stored more than 6 days at 4°C. He also demonstrated that units resuspended initially in 5% albumin medium and stored for five days at 4°C did not tolerate subsequent washing, while those suspended in plasma tolerated this step with acceptable post-transfusion survivals (Valeri 1965b). Because of these difficulties Valeri and Runck (1969b) substituted glucose-saline medium (250 ml of buffered saline solution pH 7.2 containing 50 mg glucose/100 ml) for 5% albumin solution or autologous plasma. However a significant decrease of the intracellular potassium and increase of the supernatant free haemoglobin was observed during the post-thaw storage at 4°C in such medium. Red-blood cells preserved by the low-glycerol-rapid-freeze-thaw technique in stainless steel container showed on the day of deglycerolization a supernatant haemoglobin level in the range from 102-699 mg/unit and intracellular K^+ range of 6.7 to 8.3meq/10^{12} red blood cell depending on the method of deglycerolization. On post-thaw storage for 4 to 48 hours at 4°C in glucose-saline medium these figures were further deteriorated to 360-1728 and 5.5 to 8.4 respectively. Using glucose-sodium chloride-phosphate medium red blood cells preserved by the high glycerol-slow freeze-thaw technique could only be stored for 24-hours post-processing and transfused with satisfactory[a] 24-hour post-transfusion survival. However, at the time of transfusion (24-hours post-thaw) the red-blood cells had to be concentrated by centrifugation and the accumulated free haemoglobin and extracellular potassium removed beforehand (Valeri 1974a).

FROZEN BLOOD AND HEPATITIS ANTIGEN

Australia antigen (HB-Ag) has been implicated as a cause of serum hepatitis following the transfusion of blood that contains such antigen. Incidence of serum hepatitis following blood transfusion could be reduced by the administration of blood that is devoid of HB-Ag. Following the application of frozen blood in clinical practice it was observed that the incidence of post-transfusion hepatitis was reduced. Thus in a retrospective study, Haynes et al (1960) observed the absence of homologous serum jaundice after the transfusion of 500 units of previously frozen, thawed red blood cells resuspended in 5% albumin solution which was preheated at 60°C for ten hours. On the other hand a prospective study of the transfusion of 154 units of frozen, thawed erythrocytes resuspended in autologous plasma showed the occurrence of the disease in one of the patients who received two units of such blood. Tullis and Lionette (1966) reported that no clinical cases of icteric hepatitis was observed in 2000 recipients of frozen-washed red cells which were resuspended in albumin media, but two cases occurred in the first 300 recipients of similarly processed cells resuspended in autologous plasma. In 1969 Huggins reported the absence of "clearly documented cases of homologous serum hepatitis" after the transfusion of approximately 20,000 units of blood frozen and thawed by his method. However, in these and in many other studies the observed decrease in the incidence of post-transfusion hepatitis was not based on sequential follow-up observations and the conclusions were reached before the development of the modern procedures of screening of the donor blood for HB-Ag.

In 1970 Tullis et al presented the most carefully documented data in this subject to date. In their study 1065 units of frozen, thawed washed red cells were transfused to 214 recipients who were clinically followed for up to six months after transfusion. Serum hepatitis was not detected in 110 recipients of 623 units of frozen-thawed red cells suspended in albumin medium, whilst four out of 104 recipients of 442 units of frozen, thawed blood resuspended in autologous plasma, showed histological evidence of serum hepatitis and one of them was icteric. In 1973 Huggins and Groverasmussen reported that in the period between 1963 and 1971, 88 patients with chronic renal diseases who were subjected to frequent haemodialysis and renal allograft had received 2998 units of frozen blood. Retrospective study of their clinical and laboratory records revealed the absence of clinical or sub-clinical hepatitis. Furthermore investigation of the postmortem records of 40 patients who died, failed to reveal any histological evidence of hepatitis. Examination of sera collected from most of these patients before and after transfusion and at different periods during the course of the disease showed that none of the sera had changed its reactivity.

The mechanism by which the hepatitis virus is removed or inactivated is not known. Tullis et al believed that freezing, storage, and thawing were not essential for the protective effect since red blood cells resuspended in plasma had transmitted the disease in spite of the fact that it has been treated in the same way as red cells resuspended in albumin. In fact they attributed the protective effect of their method to the so-called "transmembrane wash", accomplished as glycerol squeezes out of the cell under the effect of

their continuous-flow washing procedure. On support of this view Werch et al (1971) reported that six out of seven units of blood that were initially positive for HB-Ag became negative after deglycerolization and the antigen could be detected in wash solution in five of the seven cases.

Meryman (1974) on the other hand, believes that glycerolization and freezing are not essential for efficient elution of hepatitis virus but washing, and simple washing with a sufficient volume of isotonic saline without either glycerolization or freezing may be satisfactory.

WHITE CELL CONTENT OF FROZEN BLOOD

The extensive application of blood transfusion in the therapy of patients has led to ever increasing varieties of sensitization to different blood components. Sensitization resulting from blood transfusion is not only restricted to antibodies against red blood cells as antibodies to leucocytes, platelets and plasma protein have also been described (Kaplan 1972). In fact the most frequent sensitization seen today develops from exposure to incompatible leucocytes and/or platelets (Dausset and Colombani 1967).

In the last few years there has been significant progress in organ transplantation, this has led to a rapid development of knowledge in the field of leucocyte and platelet antigen group systems. The latter has made possible a better understanding of the immunological problems associated with leucocyte and platelet transfusion. Thus we now realize that leucocytes and platelets carry a variety of alloantigens, some of which are common to erythrocytes, leucocytes and

platelets e.g. A, B, H, M and N antigens and others shared by leucocytes, platelets and other tissues, like the histocompatibility antigens, and still others which may be restricted to leucocytes or even one type of leucocytes e.g. the lymphocytes, granulocytes or neutrophils (Dausset and Colombani 1967). The latter type of antigen (NA-1) might be responsible for the newborn neutropenia as a result of foeto-maternal alloimmunization (Mollison 1972). However the alloantigens most powerfully expressed on leucocytes and platelets are those of the HL-A system. In order to pick up a suitable donor for tissue transplantation, lymphocytes or platelets are usually used in the tests of tissue-typing. The HL-A system consists of two series, the LA and the Four series. The first contains nine alleles at the LA sublocus and the second contains 13 alleles at the Four sublocus on the HL-A chromosomal region. Because the two sub-loci are very close to each other they are always transmitted together. The combination of one LA and one Four determinant forms an HL-A haplotype. Two haplotypes are present in each individual giving him a unique genotype. There are at least 100 haplotypes and more than 8000 theoretical genotypes. One parent can only pass one of two haplotypes to his offspring so that the chance of finding two identical individuals between siblings is at least 25%. The chance of finding two identical persons among randomly selected people is low. For this reason, in renal transplantation, the best matched donor is accepted rather than waiting for an identically compatible individual.

Since leucocytes and platelets carry a variety of antigens specific to themselves, it would thus be expected that the introduction of incompatible leucocytes and/or platelets may lead to the production

of antibodies against the foreign antigens transfused.

Antileucocyte and antiplatelet antibodies do not occur naturally, they are always of immune origin produced after blood transfusion (Dausset 1954), after pregnancy (Payne and Rolfs 1958) and sometimes after tissue or organ transplantation (Thorsby and Kissmeyer-Nielsen 1968). Biochemically these antibodies are 7S gamma-globulins, active at 37°C. They can be detected by leucoagglutination (Dausset et al 1954), cytotoxicity, complement fixation or antiglobulin consumption techniques (Dausset and Colombani 1967).

The frequency of detecting leucocyte and platelet antibodies in the sera of transfused recipients increases with increasing numbers of transfusions. In general this frequency varies from 5-10% in patients received 10 transfusions or less, to 50% in those transfused 25 times or more (Brittingham 1957). However, it appears that this incidence was underestimated as isoimmunization due to blood transfusion may occur with the production of incomplete antibodies, detectable by antiglobulin consumption, rather than the complete type detectable by leucoagglutination (Van Loghem et al 1958).

Experimental studies have shown that seven or eight transfusions might be necessary to produce alloimmunization to leucocytes (Dausset and Colombani 1967). Marchal et al. (1958) have used whole blood from a single cource for the immunization of each of six volunteers. Each individual received 75 ml of whole blood from only one donor at weekly intervals for 12 to 19 weeks. After seven transfusions, four out of the six recipients developed leukoagglutinins against the donor leucocytes in their sera. Brittingham and Chaplin (1961) immunized a normal individual with fresh leucocytes from a leukaemic

patient. A leucoagglutinin was produced after 10 intravenous injections, but the survival of the transfused cells was shortened after only five injections.

The frequency of detecting leucocyte antibodies in transfused females is significantly higher than that in males. This is probably the result of alloimmunization produced by previous pregnancies (Payne and Rolfs 1960). Again the incidence among multiparous women is a function of the number of pregnancies (Mollison 1972).

Leuccocyte antibodies have also been demonstrated in sera of patient received skin grafts (Thorsby and Kissmeyer-Nielsen 1968). The cytotoxicity test is the most suitable technique for detection of the latter type of antibodies. The importance of leucocyte antibodies lies mainly in the role they play in febrile transfusion reactions as well as their presence in sera of potential organ transplant recipients can jeopardize the survival of the transplanted organ.

Hazards of transfusion of incompatible leucocytes:

A - Sensitization of the recipient against HL-A factors which may result in:

1. Development of febrile transfusion reaction on subsequent transfusion.
2. Jeopardize the survival of organ transplant.

B - In special circumstances viable lymphocyte may produce graft versus host disease.

C - Carry the risk of transmission of leucocyte-bone viruses.

TRANSFUSION REACTION

During the past two decades considerable attention has been given to leucocyte antibodies as a cause of blood transfusion reactions. Nonhaemolytic episodes represent more than 90 percent of all transfusion reactions (Heinrich et al 1973). The great majority of these are febrile and in the latter definite leucocyte antibodies could be demonstrated in most of them (Payne and Rolfs 1960). The frequent demonstration of leucocyte-alloantibodies in sera of patients with febrile transfusion reactions had originally led to the concept that these antibodies were primarily responsible for nonhaemolytic transfusion reactions (Brittingham and Chaplin 1957, Payne and Rolfs 1960, Perkins et al 1966). Indirect proof for this hypothesis came from the demonstration that febrile nonhaemolytic transfusion reactions were successfully prevented, in patients with leukoagglutinins in their sera, by administration of leucocyte-poor blood (Brittingham & Chaplin 1957, Payne and Rolfs 1960). The direct evidence was produced from an experiment performed by Brittingham and Chaplin (1957) in which one of the authors was injected by blood containing a fairly high concentration of leukoagglutinins. The recipient developed typical febrile reaction within 45 minutes of infusion.

Patients who receive repeated transfusion of whole blood for the treatment of their illness may become immunized against incompatible leucocytes in that blood. The development of these antibodies correlates with the number of transfusions (Cannon 1970). Many techniques have been developed for the detection of these antibodies e.g. leuko-agglutination, lymphocytotoxicity, complement fixation etc. (Thulstrup 1971). However it was only recently that Heinrich et al.

(1973) showed that most, if not all, of these antibodies are directed against antigens of the HL-A system. These antigens are present on granulocytes, lymphocytes, and platelets, as well as all nucleated tissue cells (Mollison 1972).

The role of leucocytes in producing a transfusion reaction in a presensitized recipient has been directly established by transfusion experiments with blood fractions. The transfusion of plasma, white blood cells or red blood cell suspensions separately showed the occurrence of transfusion reactions only when leucocytes were introduced (Dausset and Colombani 1967). This was later confirmed by the work of Thulstrup (1971) who showed that, if serum from patient with repeated transfusion reaction was mixed with leucocytes from the respective donor, incompatibility was always seen.

Perkins et al (1966) showed that the severity of febrile non-haemotytic transfusion reactions has a direct correlation with the number of incompatible white blood cells transfused. They also demonstrated that not only granulocytes but also lymphocytes and platelets be capable of inducing a febrile transfusion reaction.

The number of stimuli required for induction of leucocyte alloantibodies in a nonsensitized individual was found to be seven injections, from the same donor, given intravenously at weekly intervals (Marchal et al 1958). Apart from parous women who may react to the first or second transfusion, the number of transfusions necessary to produce febrile nonhaemolytic reactions lies between 10 and 20 when random donors are used (Payne and Rolfs 1960).

There is a wide variation in the literature concerning the

threshold dose necessary to produce a reaction in a presentized recipient. While Brittingham and Chaplin (1961) found that 0.4×10^9 is a subminimal dose, Perkins et al (1966) showed that the threshold value varies from 0.25×10^9 to more than 2.5×10^9. The last named authors have defined a significant reaction as a $1^{\circ}C$ rise in temperature over the baseline level. Nevertheless, little is known about the minimal dose of incompatible leucocytes required to sensitize a normal individual. It is also not known whether antibody formation is dependent upon the 'infusion' of intact and viable cells.

Despite the fact that febrile nonhaemolytic transfusion reactions are usually not severe, they are not desirable because of the great discomfort they cause to both the patient and physician. Moreover, the reaction may be a cause of waste of blood and results in delay in transfusion while red blood cell incompatibility is ruled out. Whilst prevention of sensitization is difficult, elimination of further reaction can be achieved by giving blood from which the buffy coat has been removed (Brittingham and Chaplin 1957).

Leucocyte transfusion and transplantation:

Patients with chronic renal failure who are potential recipients of kidney transplants are usually kept on frequent haemodialysis. During this period of maintainance the majority of these patients require repeated blood transfusion. Since leucocytes and platelets in the transfused blood carry antigens of the HL-A system which are also shared by other tissues including the kidney, it is not surprising that many of these patients become immunized against a subsequent renal allograft. The presence of antibodies in the sera of patients

directed against HL-A antigen on the kidney at the time of transplantation is usually associated with a high incidence of early graft failure (Kissmeyer-Nielsen et al 1966, Patel and Terasaki 1969). For this reason many centres have employed a compatibility test between recipient's serum and donor lymphocytes, as a measure of safety prior to transplantation. Transplantation of the kidney in spite of a positive crossmatch has resulted in an 80% failure in the early stages after the operation in one series (Patel and Terasaki 1969). If the lymphocytotoxic crossmatch is negative the existence of cytotoxic antibodies, even if they are not directed against the donor kidney, would have an adverse effect on the survival of the transplanted kidney.

The effect of pre-existing antibodies in the serum of a recipient becomes more evident when the transplanted kidney is obtained from random "cadaveric" rather than from genetically related donor (Patel et al 1971, Terasaki et al 1971). In these cases 80% failure of the graft occur in the early stages of transplantation. This was believed to be due to the multiplication effect of the graft incompatibility by the pre-existing leucocyte antibodies which was produced by multiple blood transfusion.

Leucocyte antibodies are not only detrimental to renal transplants but also to other tissue and organ grafts. Their presence at the time of transplantation may jeopardize the survival of bone marrow graft. Experimental studies with animals showed that transfusion of whole blood, whether from a sibling marrow donor or from unrelated donors, prior to marrow transplantation results in acute graft rejection (Storb et al 1972).

Hattler et al (1966) have also demonstrated that the transfusion of 500 ml of whole blood to a healthy recipient sensitized him against a skin graft from the blood donor and led to accelerated rejection of the transplanted tissue.

Leucocyte and graft versus host disease:

Animals with a deficient homograft rejection mechanism, if challenged with a sufficient dose of competent allogenic cells, develop a fatal graft versus host reaction (Hong et al 1968). A similar reaction was obtained when viable allogenic lymphocytes were administered to immunologically deficient children. However the risk of graft versus host reaction also exists in patients under severe immunosuppression (for the prevention of transplant rejection) and in patients with an acquired cellular immunological deficiency as a result of diseases. The clinical implication of this, is that when blood transfusion is considered for such patients every effort should be made to avoid the administration of large doses of viable lymphocytes with the transfused blood.

It would thus appear that the transfusion of incompatible leucocytes is undesirable and the question now is what can be done to avoid this? HL-A typing and antibody screening is not a routine technique in any red cell cross matching service and even if it is to be applied it is not infeasible in practice to transfuse identical or HL-A compatible blood. However, since in most of the cases where blood transfusion is needed the blood is given with the sole object of supplying red cells, reduction or elimination of immunization against HL-A antigens could be achieved if the erythrocyte preparation

are made sufficiently devoid of them. In fact leucocyte - poor blood has been a standard preparation in many centres for quite a long time and several methods have been proposed for that purpose.

Methods of preparation of leucocyte-poor blood

The realization that febrile non-haemolytic transfusion reaction could be minimized by the removal of the buffy coat (Brittingham and Chaplin 1957) has led to the development of various methods for the preparation of leucocyte-poor blood.

Differential Centrifugation:

This is based on the fact that the density of cells increases from platelets, to lymphocytes, to granulocytes to erythrocytes. Two different techniques could be used, either light centrifugation, in the upright position followed by expression of the supernatant plasma, buffy coat and the upper layer of the red cells or by hard spinning in an inverted position followed by removal of the red cells from underneath the buffy coat and the plasma through one of the outlet ports in the plastic bags. Both methods give more or less the same results with the removal of 70 to 80% of the white cells and loss of about 25% of the red cells (Polesky et al 1973, Miller et al 1973, Tenczar 1973, Perkins et al 1973). It has the advantage of simplicity, a minimum requirement for special equipments and using a closed system. However, the high red blood cell loss is a major drawback as this will expose the patient to the risk of multiple transfusion from additional donors in order to reach the desired level of haemoglobin. Platelets and lymphocytes are fairly

completely removed by this method but granulocytes heavily contaminate the upper layer of the packed red cells, however, the final product was found adequate for the prevention of febrile reactions in recipients sensitized to leucocytes. The fact that red cell suspensions prepared by differential centrifugation contain seven times more leucocytes per gram of red cells than products prepared by other techniques (Miller et al 1973) would imply that blood prepared by this method is more antigenic and hence unsuitable for candidates for renal transplantation.

Nylon filteration:

This method was introduced in 1962 by Green Walt et al. It makes use of the fact that granulocytes, under normal circumstances, adhere to the nylon material. The process is enhanced at 37°C temperature and at pH above 7.0 (Frey-wettstein and Bachman 1974) in the presence of heparin as anticoagulant, while it is inhibited by acid-citrate-dextrose (Halterman et al 1973). Nylon-wool filters retain virtually all the granulocytes but allow the red-cells and lymphocytes to pass through. However, the presence of the latter must be taken seriously, because the lymphocytes have a very long biological half-life and thus are immunologically competent (Miller et al 1973).

When the process of nylon-wool filteration was followed by centrifugation of the blood in an inverted position the final product was relatively pure (97% of the leucocytes and platelets are removed) but the red cell loss is considerably higher. In addition the process requires a long preparation time and the shelf-life of the

is short (Miller et al 1973).

A further disadvantage of filteration of heparinized blood through a nylon-wool colomn is the limited shelf-life, and the inability to salvage any of the blood components. Halterman et al (1973) described a method which combined nylon filteration and differential centrifugation and overcame most of the disadvantages of either technique alone. In their method blood was collected in ACD anticoagulant as usual, centrifuged and the plasma and the upper 50 ml of the packed cells removed separately. The packed cells could then be stored for up to 10 days and when required heparin and calcium chloride, necessary for phagocytosis were added. This is followed by nylon filteration which removed 97% of the leucocytes. However the method has the disadvantage of losing 30% of the erythrocytes.

Recently Frey-wettstein and Bachmann (1974) suggested that blood collected in CPD were suitable for filteration through nylon filters, even after storage for up to seven days at $4^{\circ}C$. When filteration was followed by differential sedimentation and removal of the upper 30 ml of the packed cells, the final product was relatively pure, red cell loss was about 25% and in addition plasma was salvaged for fractionation.

Diepenhorst et al (1972 a,b,c) developed cotton wool filters which retained about 95% of the leucocytes and 90% of the platelets from whole blood or red cell suspensions that had been stored at $4^{\circ}C$ for up to three weeks. After filteration the red cell recovery was over 90% and the blood could be stored for an additional week. Blood processed by this method required no heparin; ACD or CPD

blood is sufficient. Furthermore, both granulocytes and lymphocytes were removed by this process. However the filters are locally produced and not readily available.

Dextran sedimentation:

This was introduced in 1959 by Chaplin, Brittingham and Cassel and based on the agglomerated erythrocyte sedimentation principle. The method is highly efficient in removing leucocytes and platelets from whole blood or red cell concentrates that are collected on either ACD or CPD using high molecular weight dextran (M.W. 185,000) Polesky et al (1973) reported more than 90 percent removal of leucocytes and platelets while the red-cell loss was less than 10%. The remaining cells, mostly granulocytes, were intact and viable.

Although the final product appeared to be highly satisfactory as a leucocyte-poor preparation its use in practice has been hindered by the slight risk of anaplylactic reactions to the dextrans (Cannon 1970). A method based on sedimentation of the red cells by dextran 100,000 to 200,000 M.W. followed by washing the red cells in the elutramatic cell washer, to remove the residual dextran was suggested by Tenczar (1973). However, the poor red-cell recovery (65 per cent) was a major drawback. Moreover, the requirement to enter the unit during processing limited the shelf-life of the preparation due to the increased risk of bacterial contamination.

Saline Washing:

This method has been advocated by many centres as a method of preparation of leucocyte-poor blood. Washing of red cells could be

accomplished with either "batch washing" (manual or automatic) or with a continuous-flow centrifugation in one of the commercially available machines. However, washing with either method was found to be inefficient in removing the white blood cells (Cannon 1970, Tenczar 1973). Residual leucocyte content in the final preparation has been reported to be over 20 percent and 30 percent with manual batch washing and continuous-flow washing, respectively (Polesky et al 1973). Furthermore the red cell loss was relatively high so that additional transfusions may be required to obtain the desired level of haemoglobin.

Frozen blood:

It has long been assumed (though without much evidence) that frozen blood is a satisfactory leucocyte-poor preparation suitable for patients in whom the administration of leucocytes and platelets is undesirable.

The first investigators to draw the attention to the usefulness of frozen blood in the treatment of patients with repeated febrile reactions were Tullis and his colleagues (1958). They observed that in spite of the large number of units of frozen blood transfused not a single febrile transfusion reaction was reported even in those patients who used to develop a reaction with every transfusion of liquid-stored blood. With the massive increase in the use of frozen blood in the therapy of patients evidence accumulated which supported these observations (Haynes et al 1960, Haynes et al 1962, Murray et al 1962, Higgins 1965). However little information has been published about the actual amount of leucocytes present in the

frozen blood as well as the viability of these leucocytes and the antigen status of the frozen blood as a whole.

Meryman and Hornblower (1973), utilizing high-glycerol slow-freezing techniques, studied the leucocyte content in red cell suspensions processed by different automatic devices. They found that while there were modest differences in red-cell recovery in the three systems (Haemonetics, Elutramatic and IBM) the residual leucocytes in the final products were approximately the same. However they indicated that there was a wide variability in leucocyte content from one unit of blood to the other. They suggested that the glycerolization and freezing procedures damaged the white cells so that during washing, the damaged material aggregated to form a mucous-like mass which adhered to the wall of the washing chamber. Nevertheless, some intact cells remained in the final red-cell suspension and almost all of them appeared to be lymphocyte in origin.

Polesky et al (1973) compared the quality of the final product of red cell suspension from different freezing techniques in terms of red-cell recovery and residual leucocytes and platelets. Included in their study was blood frozen by the Huggins technique and deglycerolized with Huggins cytoglomerator, blood frozen by the high-glycerol slow freezing method and washed in the Haemonetics or the Elutramatic system, and red cells frozen by the low-glycerol rapid freezing technique and processed by manual batch washing according to the method of Rowe et al (1968). They found that although all these methods were equally effective in removing the platelets, there were marked differences in leucocyte removal in each of the four systems. Best results were obtained with continuous-flow washing (whether in

the Haemonetic or Elutromatic systems) where the red-cell recovery was around 90 percent and the residual leucocytes were 1.8 $\pm$ 0.8 percent. Next in efficiency was the Rowe technique where the red cell recovery was 92.2 $\pm$ 8.0 percent and the residual leucocyte were 5.3 $\pm$ 1.8 percent. The Huggins method gave the highest leucocyte contamination (17.1 $\pm$ 8.4 percent) and the lowest red cell recovery (86 percent). However when blood frozen by Huggins' technique was deglycerolized by methods other than Huggins Cytoglomerator the leucocyte removal was improved. The authors also found that most of the residual leucocytes were unidentifiable smudges and basket cells and when the viability of these cells was tested by the trypan blue exclusion test, all of them were non-viable. It would therefore, appear that freezing techniques are the most promising methods for preparing leucocyte poor blood. However, Helgeson et al (1973) have pointed out that although no viable leucocytes were recovered from frozen blood, serious problems of residual cell fragments with antigenic activity may limit the effectiveness of these preparations in preventing immunization of the recipients.

Crowely and Valeri (1974) utilizing the high-glycerol slow-freezing technique, suggested that glycerolization caused most of the damage which occurred to the white cells and that during washing in the Haemonetics-disposable bowl, the damaged cells agglomerated in large masses which were either washed out or remained adherent to the washing bowl. They maintained that these events effected mainly the polymorphnuclear granulocytes, as 70 percent of the remaining cells were lymphocytes. When the viability of the latter was tested by their ability to exclude the trypan blue dye, most

of them appeared to be viable. Also HL-A antibody adsorption tests showed that particulate antigens were still present in the previously frozen red-cell suspension after washing, but these antigens were removed completely after passing the blood through a micropore filter.

It is thus evident that in spite of the fact that most of the work conducted in this subject was performed on blood frozen by the high-glycerol slow-freeze technique, the data obtained from different laboratories is conflicting. At the same time no information is available on the leucocyte content of blood frozen by the low-glycerol rapid-freeze-thaw technique in aluminium cans. For this reason this subject will constitute a major item in this study.

MATERIALS AND METHODS

DESCRIPTION OF THE SYSTEM

The system adopted for freeze preservation of blood in Edinburgh Blood Transfusion Service is an adaptation of that system used by Jenkins and Blagdon at Brentwood (1971) which is itself derived from the work of Krijnen in Holland (1964) and Pert in the U.S.A. (1963, 1964, 1965). The system is a low glycerol-liquid nitrogen-aluminium can system.

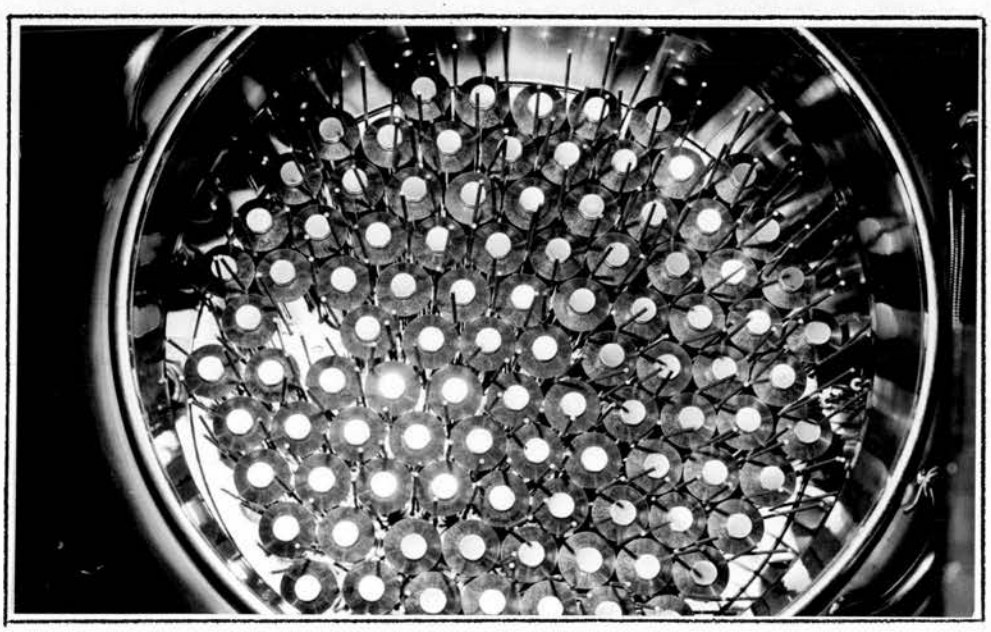
The liquid nitrogen is stored in a BOC TWN/500/1 horizontal tank having a liquid capacity of 500 litres. As supplied, the tank is unsatisfactory and a number of modifications have been introduced to it. The contents are indicated directly by a load cell (Darenth-Helios Hydraulic). The pressure gauge was replaced with a 0-60 psi 7" gauge; remote mounted. The safety relief valves were replaced by "Circle-Seal" valves. The bursting discs were replaced with 47 psi aluminium discs.

Experience has shown that no automatic filling system can operate reliably below 25 psi and thus the tank pressure is always maintained at 30 psi. Experience has also shown that no design of safety relief valve will reliably re-seal when cold and they should thus be mounted on extension pipes.

The boil off from this tank is allegedly 5 litres per day, but this does not include the liquid consumed in pressure raising.

The liquid is fed into the building through a half-inch brazed copper pipe insulated to 6" diameter with polyurethane foam. The

(Fig. M 1.)



(Fig. M 1.) top view.

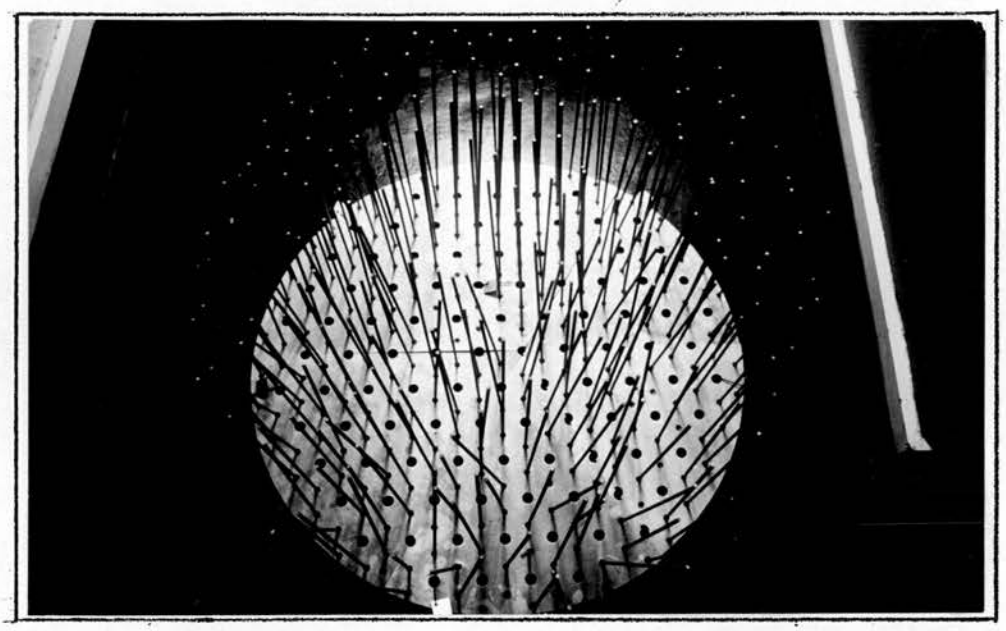
filling time, similarly constructed is fed into the tank outlet via a tee-piece and extra valve.

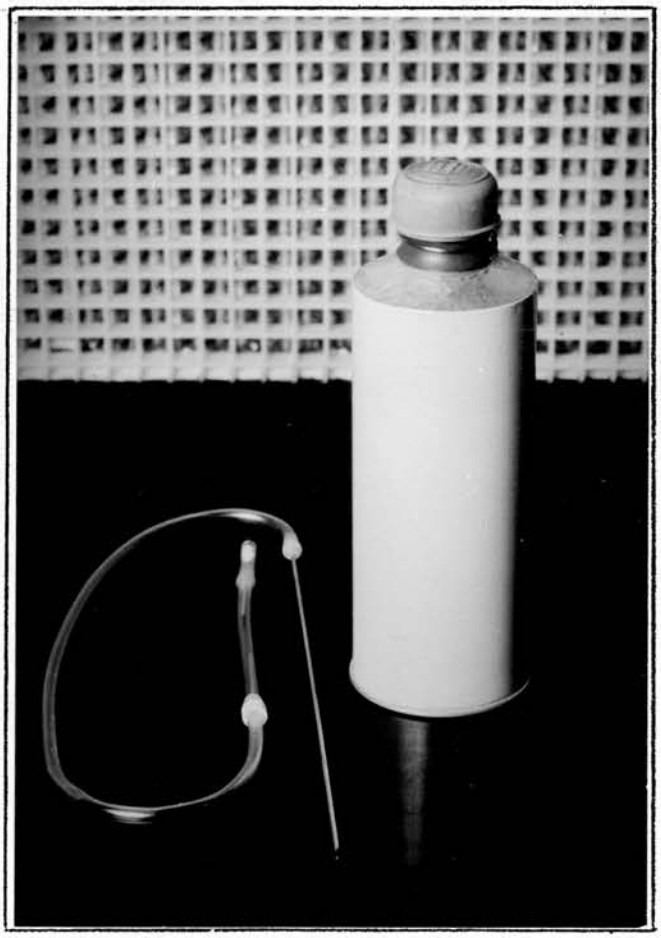
The refrigerators used are Union Carbide 250 litre models (Fig. M.1). These are cylindrical, open top steel Dewar vessels with split foam tops providing either half or full opening. These refrigerators are very convenient to use, as the operator can reach to the bottom without difficulty. The filling from the manifold system is performed via electrical solemoids of eight-inch orifice, these have proved to be very reliable.

The level of liquid nitrogen is maintained just below the false floor of the refrigerator by automatic level sensors. In addition, a third emergency low level alarm sensor is fitted. The storage area of the refrigerator is thus operated entirely in the vapour phase which has important bearing on the safety of operation. The temperature in the top and bottom of an empty refrigerator has been measured and found to be $-188^{\circ}C$ at the floor and $-175^{\circ}C$ at the top. Paradocically, when full, a refrigerator is colder than when it is empty. This is due to the increased "heat leak" provided by the blood cans, causing more nitrogen to boil up from the base reservoir and cool the upper section. In practice, we find that the refrigerator switch on the supply approximately once every 24 hours and that each refrigerator consumes 12 litres per day. Added to this are 10 litres per day lost in the transfer lines giving a total weekly consumption for five refrigerators of 490 litres.

The refrigerators are divided up by vertical rods in a hexagonal pattern which allows for the close packing of cans (Fig. M.2) two layers can be filled, with 102 cans per layer. The can used in this

(Fig. M 2.)





(Fig. M 3.)

system (Fig. M.3) is slightly smaller than that previously developed (Jenkins and Blagdon 1971) and thus takes up less space for the same amount of blood. The can also has a narrower internal neck diameter with convolutions which serve to give a better seal against the ingress of nitrogen. These cans have been pressure tested to destruction and the following sequence of events was observed:- At 80 psi the concave base everted into a convex base, and at 100 psi the base was blown cleanly off and downwards, the rest of the can and seal were left intact. It is therefore believed that this is an inherently safe can since the seal at the top does not fail, the operator is given warning 20 psi before failure and the can base which is smooth and light is blown downwards away from the operator. Furthermore, since the sides of the can are not ripped open, no jagged metal is exposed. Finally this can is of British manufacture and costs approximately half of the imported Printal can from Finland.

Preparation of cans for freezing:

Cans are soaked in 1% V/V alkaline laboratory detergent (RBS 25) in hot water for not more than 15 minutes. Prolonged soaking might lead to subsequent leaching out of aluminium into the blood.

Cans are next rinsed in 0.2% V/V acetic acid to neutralize the alkaline detergent. Contact time should again be limited.

Rinse three times in distilled water and three times in hot pyrogen free water. Cans are then dried in an air oven at 110°C. Two ml of pyrogen free water are dispensed into each can. This is preferred over saline to minimize the leaching out of aluminium. The neck is sealed with a boiled Suba-Seal No.57. The latter is moistened with glycerol and pushed in until four rings remain visible.

The seal joint is then liberally smeared with phenol-glycerol reagent and the skirt immediately turned down and wired into place with two turns of 1.6 mm enamelled copper wire. The wire seal has now been replaced by a shrink fit TFE (Dupont $1\frac{1}{4}$" roll cover) approx. $\frac{3}{4}$" length. During autoclaving this shrinks tightly around the cap. The upper surface of the seal is marked in a horizontal line with a felt-tipped marker. Can seals are covered with temporary dust seals of two paper cake cases held in place with elastic bands (Fig. M.4).

The cans are then weighed on a top pan balance to within 0.1g before and after autoclaving. The observed weights are noted on the base of each can. The cans are autoclaved at 121°C for 20 minutes. The presence of the water inside the can generates a differential pressure of about 35 psi which is a good test of assembly. The whole process should be completed in six hours. After autoclaving the cans are reweighed and any cans which have decreased in weight by more than 0.5g from that prior to autoclaving are discarded.

COLLECTION OF BLOOD:

400 ml of blood is collected from healthy volunteers in routine sessions into plastic packs containing one of the following anti-coagulants according to the need of the department.

a - 70 ml ACD from Tuta, Lane Cove, N.S.W. Australia.

b - 70 ml CPD (Tuta, Laboratories Australia).

c - EDTA (Tuta Laboratories Australia).

Plasma is separated after centrifugation at 1,300g for 60 minutes and the packed cells are stored at +4°C for not more than one week.

GLYCEROLIZATION:

During the early period of this study we were interested in finding out the glycerol concentration that gives the highest red cell recovery. Therefore it was essential to determine the volumes of the glycerolizing solution and that of the blood used each time. The volumes were worked out from the weights and the density of each substance and the observed readings were recorded in special form.

In this study blood of any group and genotype was used, however, for routine storage we use only group O blood of genotype R_1R_1, R_2R_2 or rr. Only packed red cells, less than one week old, were frozen. The red cells were prepared from blood donated in routine sessions and collected on ACD, CPD or EDTA.

METHOD:

The red cell pack (including a plasma transfer line), the Freezing Protective agent (FPA) bottle and the aluminium can are weighed to the nearest 0.1g on a top-pan balance (Mettler P 1000 N). The plastic pack containing the red cells is connected via the giving port to the plastic spear of the transfer line.

With the clip of the line closed, the needle at the other end of the line is entered into the half-MRC bottle containing the FPA. An airway needle is also introduced into the bottle. The blood pack is placed on horizontal Luckhams Shaker and the FPA bottle is hung from its base above it as high as possible (Fig. M.5). The shaker is switched on at a rate of 200 rpm and the line clip is opened to allow the FPA into the blood pack. When the FPA is completely transferred

(Fig. M 4.)

(Fig. M 5.)

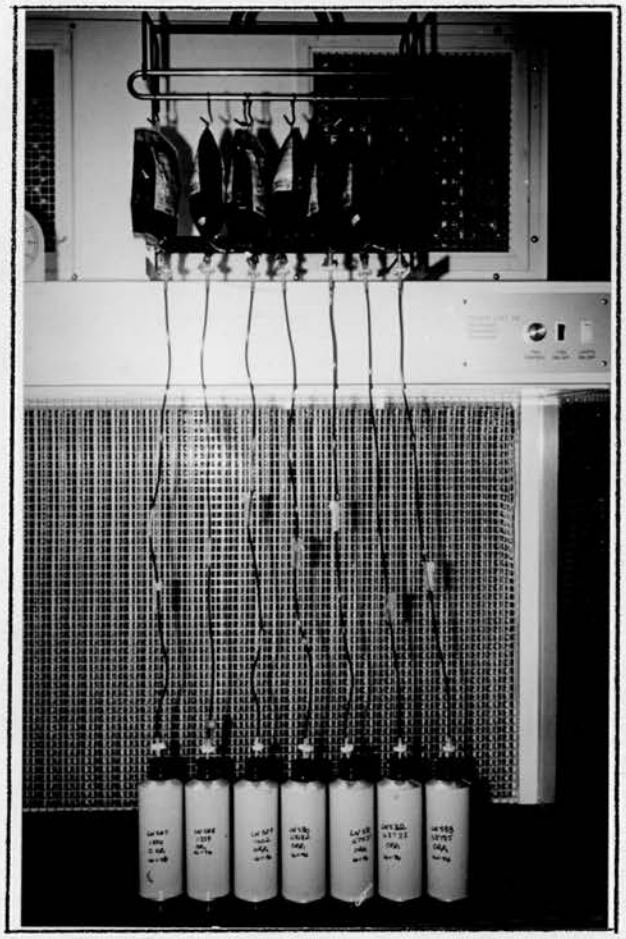
into the plastic pack the line clip is closed and the pack is disconnected from the FPA bottle. The full pack and the empty bottle are reweighed. The glycerolized blood is then transferred into a prepared aluminium can (Fig. M.6). Since the puncture seal is well maintained when the smallest possible gauge is used, we preferred to fill via 19 SWG needle and use a 21 SWG needle as an airway. The puncture site on top of the Suba-seal is marked with a felt-tipped pen line so that entry needles puncture along the line and emptying needles across it. This avoids the accidental entry of two needles at the same site.

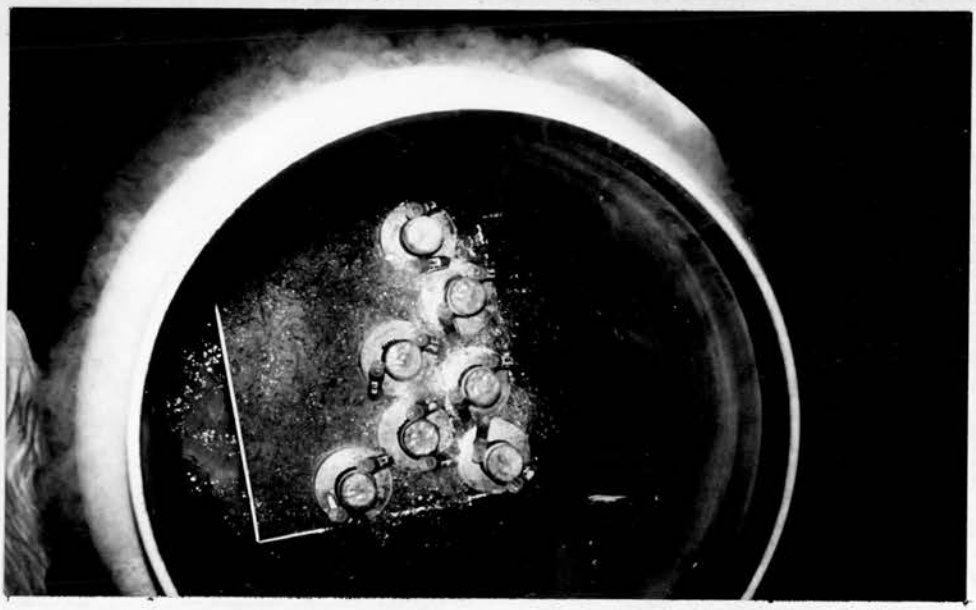
Whilst the blood is running into the can, and before the line is disconnected, the can and pilot tubes must be labelled with the details of the unit. A record of freezing file card is also made out and indexed by the "liquid nitrogen - number" LN - this number also appears on the can side and base and pilot tubes. The record card is also made out with a full genotype of the blood as far as the availability of antisera permit (The latter information was obtained from the serology laboratory).

When the can is filled, the remaining blood in the transfer line is expressed with a tubing stripper to provide two aliquots of one ml for each of the two pilot tubes. The plastic bag is then disconnected from the can and both are reweighed.

The upper seal surface is smeared with phenol-glycerol mixture, the latter serves as both bacteriostatic and cryogenic seal. Two finger cotts (latex rubber) are placed over the top seal and the two pilot tubes are held in place by a thin copper wire which passes through the body of the pilot tube and is tied around the neck of the can.

(Fig. M 6.)





(Fig. M 7.)

FREEZING:

Cans are frozen by direct immersion in liquid nitrogen. Single cans are frozen in 4.5 L Dewar flask whilst up to 12 cans may be frozen simultaneously in a Union Carbide LNR 40 refrigerator (Fig. M.7). Care should be taken to keep the liquid level up to the can shoulder during the rapid stage of boil-off.

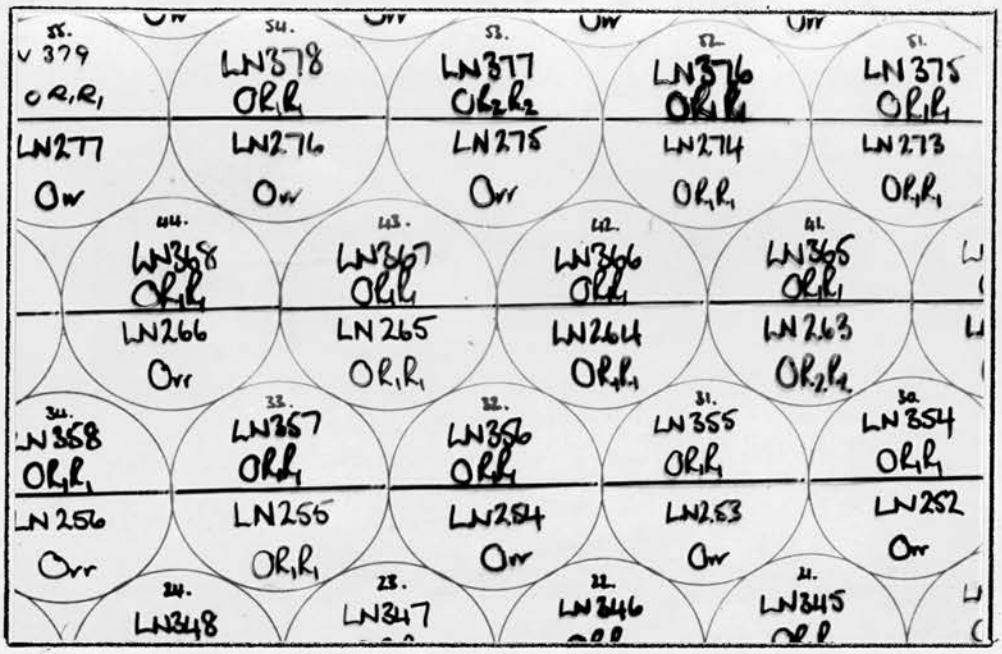
When the liquid nitrogen stops boiling vigorously, usually in 10-12 minutes, the cans are removed and transferred to the storage refrigerator. Under no circumstances should unfrozen cans be placed directly in the storage refrigerators.

The LN number, the blood group and genotype details are entered onto a wall map (Fig. M.8), which is a full size replica of the refrigerator interior. Arranged on the map in hexagonal close packed array are the circular sites representing 102 positions. Since two layers can be placed, the total refrigerator capacity is 204 units and so each map position is divided horizontally to represent the upper and lower sites (Fig. M.9). The under surface of the map is a reproduction of the plan showing can sites and position codes only. The over surface is of transparent map-covering acetate film and it is on this surface that the details of each can are entered. If a felt-tipped marker is used, it may be cleaned off with chloroform.

THAWING:

When required the desired unit of blood is retrieved, the pilot tube is detached and thawed in a water bath at 37°C. The contents are transferred to a conical 10 ml centrifuge tube and one ml of prewarmed 19% W/V sorbitol saline is added. After one minute of equilibration

(Fig. M 8.)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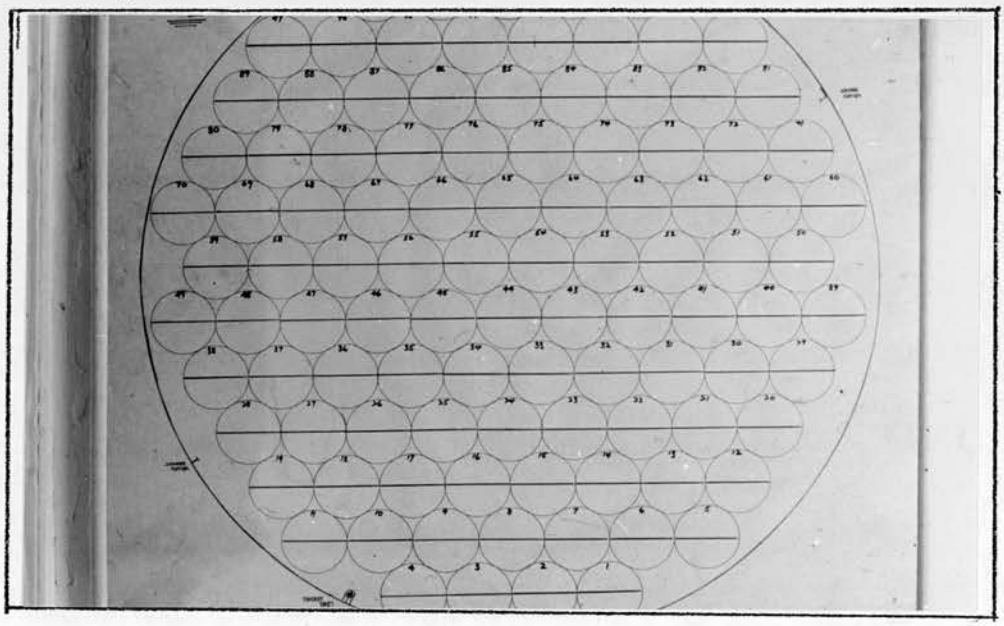


(Fig. M 9.)

a further 8 ml of prewarmed saline is added with continuous mixing. The resulting mixture contains 10 ml of 3% V/V cells in 2% glycerol and 2% sorbitol. This mixture can be used directly for cross-matching without any further interference, however, if desired the cells can be centrifuged and resuspended to give a more concentrated sample.

In order to avoid any confusion resulting from mixing up of pilot tubes, we preferred to use samples collected directly from the processing bag for cross-matching. This sample is collected immediately after washing is completed.

Assuming a negative cross-match, the required can is then taken out of the refrigerator using 18" crucible tongs and industrial asbestos gloves (the latter are not impermeable to liquid). Single or double units can be thawed in a sink of hot water at 37-41°C by continuous shaking at about one cycle per second. However, for convenience in handling, up to four cans may be thawed in the gyratory action thermostated washing machine (the latter is home made and was developed after Mitchell et al 1973). This has the additional advantage that any hazard from exploding cans is much reduced by the machine lid.

The completion of thawing is detected by the absence of "sloshing" inside the can and/or the absence of a cold sensation when holding the can. This takes from six to ten minutes. In no case should the cans be allowed to warm up to more than 40°C as the rate of haemolysis is very high at this temperature.

DEGLYCEROLIZATION:

This may be performed either manually or automatically (in the IBM 2991 Automatic Cell Processor). In either case the washing is a serial centrifugation or batch washing.

MANUAL DEGLYCEROLIZATION:

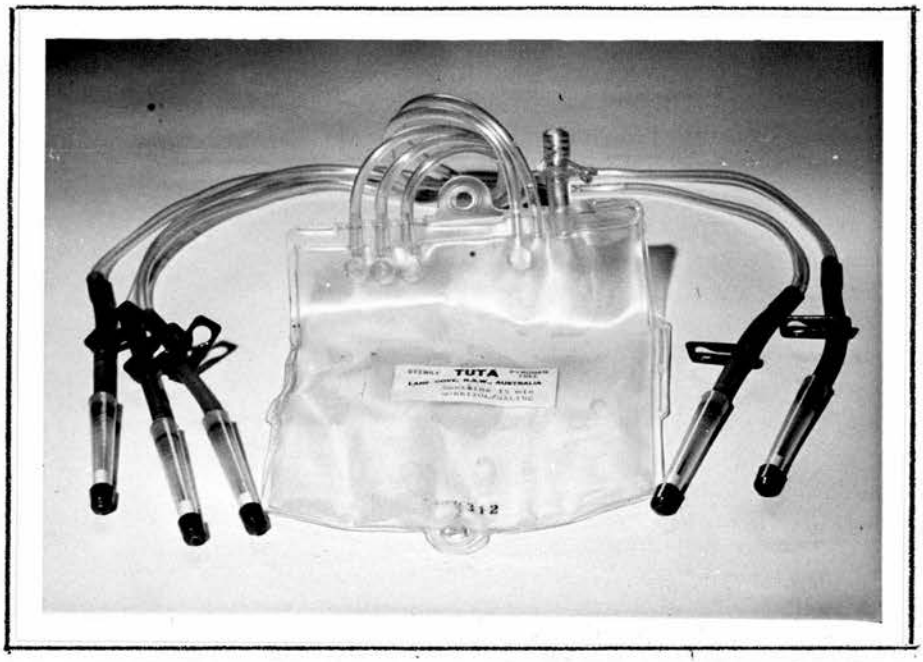
Prepare four dry clean MRC bottles and mark them as follows

0	-	post thaw supernatant
1	-	wash I "
2	-	wash II "
3	-	wash III "

Get ready a Tuta five-tail washing pack (Fig. M.10) and with a felt-tipped marker enter the details of the thawed unit on its surface. The pack is placed on a Luckhams shaker. After thawing, the can is clamped in an inverted position at about 15" high above the shaker, the outer elastic covers are removed, the outer surface of the Suba-seal is swabbed with iso-propanol and the first line of the five-tail pack is entered into the Suba-seal. Any gas pressure is now manifested by the expulsion of a volume of blood prior to airway entry. If the volume exceeds 10 ml then the can seal was not perfect. We have had several leaks when using conventional British long airways and have found the thinner needles of plasma aspirating sets to be more satisfactory. Both Baxter ($6\frac{1}{2}$") and Avon (9") sets have been used.

Having filled the pack via the first line, this is either clipped off or heat sealed and cut off. The bag is then centrifuged at

(Fig. M 10.)



(Fig. M 11.)

2500 rpm in a swing out rotor centrifuge for ten minutes. The viscosity of the glycerol dictates the longer spin time.

Using a Renwal plasma expressor, the post-thaw supernatant is expressed via line 2 into the MRC - O bottle and then wash I solution (Sorbitol) is allowed to run in via line 2. At this stage the shaker should be operating to ensure thorough mixing of the pack content and the incoming wash solution to avoid hypertonic stress to the cells. The effect of the sorbitol is to shrink the cells and "wring out" any residual glycerol and these cells are thus much smaller and denser.

Line 2 is then cut off and the pack is again centrifuged at 2500 rpm but for five minutes only this time. The cells pack down very hard and wash I supernatant is expressed via line 3. Wash II (0.9 per cent NaCl Solution) is run in through line 3 whilst the pack is on the rotary shaker. It is at this stage that most haemolysis takes place for the shrunken cells are exposed to an expanding medium and care should be taken that all the "sludged" cells at the corners and edges of the pack are resuspended.

Line 3 is cut off and the pack centrifuged at 2,500 rpm for five minutes. Wash II supernatant is expelled via line 4 and wash III (also isotonic saline) is run in via line 4 whilst the pack is on the rotary shaker.

Line 4 is cut off and the final spin is again performed at 2,500 rpm for five minutes. The wash III supernatant is expressed via line 5 and the desired resuspension medium is introduced via line 5 to give the required haematocrit.

The choice of swing out versus angle rotor depends on the time factor, processing in a swing out rotor (as described) is slower but gives a cleaner interface. Angle rotors are faster but the cell-supernatant interface, especially after wash II, is very hard for inexperienced operators to see.

The whole process takes about 90 minutes, and up to four units of blood can be processed simultaneously.

AUTOMATIC WASHING IN THE IBM SYSTEM

Similar to manual washing the wash process utilized in the IBM 2991 Red Cell Processor is discontinuous and serial. Unlike the manual washing the IBM wash system is a closed and automated system.

Description of the system: (Fig. M.11)

A plug board at the front of the machine allows the presetting of the number of wash steps as required and the order of wash solutions. Up to seven wash cycles can be programmed during which up to three different wash solutions can be applied. Dials permit the selection of the speed of spinning of the centrifuge bowl as well as the length of time of each spin operation.

Blood enters the system by gravity through one of the tubing lines and is collected into the processing bag in the centrifuge bowl. The processing chamber is a circular plastic bag which is integrally connected to a single-channel rotating seal and a tubing harness. The latter connects the processing bag to the blood, wash solutions, and supernatant collecting containers. The bag and its related seal

and harness are disposable and supplied as an integrated, sterilized unit.

The centrifuge bowl can be spun at any required speed, up to a maximum of 3000 rpm (1250g), by presetting the corresponding rotary switch. Separation of cells and cell-supernatant interface are visible during spinning through a transparent centrifuge cover and bag. This allows intervention of the operator at any stage should anything go wrong.

A manual prime step is necessary to drive the air out of the system after which the various steps of wash cycle (centrifugation, removal of the supernatant, addition of a new wash solution and mixing) take place automatically and are preprogrammed.

When the spinning-time ends a valve to the supernatant-collect container is opened and a pump starts forcing hydraulic fluid under the flexible membrane in the centrifuge cavity. This squeezes the supernatant out to the container. When the erythrocytes appear at the red cell detector, the pump and centrifuge bowl stop and a preselected wash valve opens, allowing wash solution to flow into the blood processing bag. As the wash solution flows in, it is mixed with the red cells by a gentle reciprocating agitation cycle of the centrifuge bowl. At the end of this cycle, the bowl again spins at the preselected rate and time interval and the cells are again separated from the supernatant. This cycling continues until the present cycles are completed.

This system can also be operated manually. Manual operation is similar to that described above except that the operator at each step has to select and initiate the wash solution to be mixed with the

red cells. Push buttons and rotary selector switches at the front board of the machine permit manual operation.

Operation of the system:

Previously frozen-thawed red cells are deglycerolized in the IBM 2991 Automatic Cell Processor according to a wash protocol developed in our laboratory to suit our freezing programme.

By the time the frozen unit is thawed, the machine is set up for operation. The tubing harness is mounted in the solenoid pinch valves, the integrally connected blood processing bag is placed in the centrifuge bowl and the plastic spikes are attached to the wash solution containers. The machine is programmed so that the red cells receive three wash cycles each oonsists of spinning for two minutes at 3,000 rpm,removal of the supernatant at a rate of 450 ml per minute, addition of a new wash solution together with mixing at an appropriate speed to allow thorough mixing.

Once the unit is thawed, it is connected to the system through the corresponding line and blood allowed to run into the processing bag. When the transfer of blood is completed, air is removed from the system and wash I is allowed to flow into the processing bag until it is completely filled. We found that this step is necessary not only because it allows for the difference between blood volume (400 ml) and bag capacity (650 ml) but also it gives better washing and higher red cell recovery. The machine is then switched to automatic operation to run according to the presetted programme.

After the separation of the post-thaw supernatant the red cells

are given one-cycle wash with wash I (19 per cent sorbitol in saline) followed by two cycles with 0.9 per cent sodium chloride.

At the end of the last spin, the supernatant is expelled and the cells are resuspended in the desired resuspension medium. A sample of the final product is then expelled, through the extra line left unused, for bacteriological, haematological and biochemical tests. The time required to process one unit in the machine is about 20 minutes.

For the sake of study the tubing line leading to the supernatant collect chamber was severed past the corresponding valve and the supernatant from each wash step was collected separately into a dry clean measuring cylinder for biochemical analysis.

The blood is sent to the wards for transfusion in the circular processing bag after sealing off the rotating seal with its harness lines (Fig. M.12).

(Fig. M 12.)

Suppliers of Equipment

Refrigerators:

Union Carbide 250 litres vivostats with control units. Supplied by Union Carbide Cryogenic Division, Redworth Way, Ayecliffe, Co. Durham.

Liquid Nitrogen Storage:

a. Portable, self pressurising 160 litre Dewar flasks (LS-160) supplied by Union Carbide.

b. For larger volumes, TWN-500-1 horizontal 500 litre storage tank, with mannual pressure raising circuit. Supplied by British Oxygen Co., (BOC) cryoproducts Division, Manor Way, Crawley, Surrey.

Freezing Containers:

a. For single cans, 4.5 litre glass Dewar flasks (With polypropylene covers) from Day-Impex, Earls Colne, Essex.

b. For up to 12 cans a Union Carbide LNR-40 refrigerator (40 litres, wheel base) is used.

Aluminium Blood Cans:

Metal Box Co., 69 mm by 160 mm, 485 ml capacity, screw cap aluminium bottles with white exterior lacquer supplied in boxes of 70. From the Metal Box Co., Cowlairs Industrial Estate, Glasgow N1.

Rubber Seals:

Stoppers are Suba-Seal No.57 with turn-over skirt. From W. Freeman, Stain Cross, Barnsley, Yorks, in lots of 100.

Pilot Tubes:

2 ml polypropylene tubes with screw caps. Manufactured by Nunc of Denmark and sold by Sterilin and Jobling-Vestric in the U.K.

Rotary Shaker:

Luckhams "Rotatest" from Luckhams, Burgess Hill, Surrey. A custom fabricated plate with retaining straps is required to take 4 packs.

Airways and Transfer Lines:

Baxter AE-2 or AE-8 plasma transfer lines. Baxter BR-325 plasma aspirator sets are used as long airways, needle length 6½". Avon plasma aspirating set D-80 also used as long airway, needle length 9".

Plastic Washing Bags:

Tuta five-tail washing packs (16-050), from Tuta plastics (UK) Ltd., 242 Tolworth Rise South, Surbiton, Surrey, England.

RESUSPENSION

At the end of the washing process the red cells may be resuspended in the required medium. When the cells are processed by the manual technique the resuspension medium is added via the line number "5" of the five-tailed processing bag, which is then cut off. With the IBM washer, resuspension medium may be added through the unused line of the processing set, while the latter is still in place, the contents are then thoroughly mixed by reciprocal agitation.

In our investigation for a resuspension medium that gives the best post-thaw stability, the following method of resuspension and sampling was used.

After deglycerolization the packed red cells were divided into two equal parts, 100 ml each, and distributed into two sterile, dry transfer packs (200 ml capacity (Tuta 16-050)). This distribution was aseptically achieved by using a three-way tap (Pharma-Seal Inc, K75) and a 50 ml disposable syringe. Both portions were then resuspended to a haematocrit of 45% $\pm$ 5%, one in isotonic saline, which served as a control, and the other in the medium under investigation. The following media were investigated:

a. Human Serum albumin solution 5 per cent (W/V in 0.9 per cent sodium chloride). This was supplied sterile from the protein fractionation centre.

b. Sodium chloride solution 0.9 per cent (W/V) to which is added 70 ml of a solution containing:

Disodium Citrate	2 g
Dextrose	1.7 g
Water to	70 ml

c. Human serum albumin solution 5 per cent W/V in isotonic saline to which is added 70 ml of the ACD solution as in "b".

d. Sodium chloride solution 0.9 per cent + 70 ml of a solution which contained:

Citric Acid	0.327 g
Sodium Citrate	2.630 g
Mono sodium phosphate	0.222 g
Dextrose	2.550 g
Water to	100 ml.

e. Human serum albumin 5 per cent in isotonic saline + 70 ml of the same solution as "d".

f. Ringer Lactate-solution (Baxter) Each 1000 ml contains:

Sodium chloride	6.0 g
Sodium lactate	3.22 g
Potassium chloride	400 mg
Calcium chloride	270 mg

g. Tis-U-Sol solution (Orlina et al 1972) which was composed of

Potassium chloride	5.8 m Molar
Sodium chloride	137.0 m Molar
Magnesium Sulphate	1.6 m Molar
Disodium phosphate	0.4 m Molar
Glucose	100.0 mg%

After resuspension the following samples were withdrawn and every effort was made to maintain sterility.

1. 10 ml sample into a 10 ml calibrated conical TPX centrifuge tube which was spun in MSE Super-Minor centrifuge for 30 minutes at 3000 rpm for determination of the packed cell volume. The latter was used for calculation of the intracellular potassium as it will be described later.

2. 10 ml sample into 15 ml conical TPX centrifuge tube which was spun for 10 minutes at 3000 rpm for separation of the supernatant fluid. The latter was used for estimation of free haemoglobin and potassium.

3. Five ml samples for estimation of blood pH, total blood haemoglobin total potassium, 2,3 DPG and ATP content.

4. A sample of resuspended blood, into EDTA sequestrene tube for estimation of:
R.B.C., W.B.C., PCV, MCV, MCHC and for making smears for haematological examination.

5. A sample of 10 ml was taken every day for nine successive days into a 15 ml conical TPX centrifuge tube for separation of the supernatant as described in "2" for estimation of free haemoglobin and potassium. During this period the blood was stored in a conventional refrigerator at $+4^{\circ}C$ without mixing apart from sampling.

PREPARATION OF SOLUTIONS

Freezing Protective Agent (FPA)

Glycerol BDH (Analar)	450 g
Sorbitol BDH	37.5 g
Sodium Chloride BDH(Analar)	9.0 g
Water to	1000 ml

Because of the physical nature of the reagents it is convenient to weigh all the constituents separately in plastic beakers and transfer with rinsing into a final large mixing vessel which is calibrated to the desired volume.

It is not normally necessary to filter this reagent, however if

dust is seen to be a problem it must be removed in the way as set out below for Wash I. As a check on the correctness of making up, the batches should be checked to see that the refractive index is 1.395 at 20°C. The solution is then dispensed in 200 ml volumes in half MRC bottles and autoclaved, at 121°C for 20 minutes.

Wash I solution:

Sorbitol	190 g
Sodium chloride	9 g
Water to	1 L.

This solution often contains visible dust and we always filter this on-line when filling the bottles. This is done by pumping the solution through a Whatman Gamma - 12 glass fibre filter of perosity 8 microns. The filtered solution is pumped through the filter and directly into the bottles by a Glen Creston variable speed peristaltic pump.

The solution is dispensed either as 300 ml in half - MRC bottle for manual batch washing or as 750 ml in one litre MRC bottles for automatic washing in the IBM 2991 processor. The bottles are then autoclaved at 121°C for either 20 minutes (for the small bottles) or 30 minutes for the 1L MRC'S.

Wash II and III

Normal physiological saline (NaCl 0.9g per cent) dispensed either in half - MRC bottles are 300 ml volumes or in 1L MRC as 1000 ml volumes. During the course of the study we shifted to commercial sodium chloride solution supplied in Viaflex bags by Baxter. The latter provided an extra precaution against bacterial contamination as an airway needle is not required.

Phenol-Glycerol-Bromophenol Blue

To 100 ml of glycerol in a glass beaker add 1g of phenol and 5 mg of bromophenol blue. The mixture is best dissolved by placing it on top of a heated magnetic stirrer with a few drops of glycerol underneath the beaker. This solution is most conveniently dispensed in 150 ml conical tipped polythene dropper bottles (Azlon).

HAEMOGLOBIN MEASUREMENT

A. The Cyanmethaemoglobin Method:

This method was used for blood samples and supernatant fluid containing haemoglobin level more than 60 mg/dl. The procedure followed was the same as described by Dacie and Lewis (1970).

Principle:

If a solution containing potassium cyanide and potassium ferricyanide is added to a haemoglobin containing solution, the haemoglobin, methaemoglobin and carboxyhaemoglobin are all transformed to cyanmethaemoglobin. The optical density of the latter can be conveniently measured at 540 nm in photoelectric colorimeter.

Reagent:

This was locally prepared and contained

Potassium ferricyanide (M & B)	200 mg
Potassium cyanide BDH (Analar)	50 mg
Potassium dihydrogen phosphate (H & W) Analar	140 mg
Triton X-100 BDH	1 ml
Distilled water to	1 litre

This reagent was kept at room temperature for up to six months in dark-coloured glass container.

Method:

One ml of the supernatant fluid or 0.02 ml of blood was added to 4 ml of the reagent solution into dry clean test tube.

The content of the tube was mixed by gentle inversion for a few seconds and left at room temperature for at least five minutes for

the reaction to be completed.

The optical density was then compared to that of the contents of a cyanmethaemoglobin standard (BDH) ampoule, at 540 nm in an EEL - SPECTRA 60 colorimeter using reagent solution as blank.

Calculation:

Haemoglobin concentration mg/dl =

$$\frac{\text{OD. of the test sample}}{\text{OD. of the standard}} \times \text{conc.of the standard(mg/dl)X dilution factor}$$

When the colour of the cyanmethaemoglobin of the test sample is too dense to be read, it was further diluted with reagent solution and the resulting O.D. was multiplied by the dilution factor.

B. The Ortho-tolidine method:

This method was used for estimation of haemoglobin levels in supernatant fluids and plasma below 60 mg per dl. The procedure followed was the same as described by Lewis (1965).

Reagents:

1. Ortho-tolidine reagent:

Glacial acetic acid (BDH Analar) 90 ml is added to 10 ml of distilled Water, covered and left overnight at room temperature.

0.25g orthotolidine is dissolved in the acetic acid prepared in the previous step. The reagent is kept in a refrigerator at $+4^{\circ}C$ and should be prepared freshly every eight weeks.

2. Hydrogen peroxide:

1.2 ml of 100 volume % solution of hydrogen peroxide was pipetted into a 100 ml flask and the volume was completed up to the mark with distilled water. This reagent is kept in a refrigerator at +4°C and should be prepared freshly every week.

3. Haemoglobin standard solution:

Supplied by BDH.

4. Acetic acid diluent:

In a one litre measuring cylinder 100 ml of glacial acetic acid was made up to one litre with distilled water.

Method:

Three 15 ml glass test tubes are prepared for each unknown and marked TEST, STANDARD and BLANK. Into these tubes the following reagents are pipetted (mix by swirling after each addition).

	TEST	STANDARD	BLANK
Ortho-tolidine reagent	1ml	1ml	1ml
Unknown	0.02ml	-	-
Haemoglobin standard	-	0.02ml	-
	Leave for 2 minutes at room temperature		
Hydrogen peroxide solution	1ml	1ml	1ml
	Leave for exactly 10 minutes		
Acetic acid diluent	10ml	10ml	10ml

2. The contents are then mixed by inversion of the tubes, transferred to 10 mm light path cuvettes and the optical density is immediately read in photoelectric colorimeter at 630 nm using the blank as reference.

Calculation:

$$\text{Hb concentration mg/dl} = \frac{\text{O.D. of the test}}{\text{O.D. of the standard}} \times C_s$$

C_s = concentration of the standard (mg/ dl).

TOTAL AND FREE POTASSIUM ESTIMATION

Immediately after washing, before the addition of the resuspension medium, the following samples were collected:

a. A sample of 10 ml collected into 10 ml conical TPX centrifuge tube. This was spun for 20 minutes at 3000 rpm in MSE Super Minor centrifuge and the haematocrit was read and recorded. A sample of the supernatant was used for estimation of the free potassium.

b. A two ml sample of blood in a plain tube for estimation of total potassium in whole blood, and for red cell count, by a Coulter Counter model "S".

Potassium in the supernatant and whole blood was estimated by flame photometery (Corning Eel 227 Flame Photometer).

Intracellular potassium content was calculated and expressed as m mol per 10^{12} red blood cells from the formula:

$$\text{conc. of K per } 10^{12} \text{ RBC} = \frac{K_c}{R} \times 10^{12}$$

where

R = red blood cell count per ml.

K_c = conc. of K. per ml of R.B.Cs and derived from the formula:

$$K_c = \frac{K_w - K_f}{10^3}$$

where K_w = conc. of K^+ in whole blood

K_f = conc. of K^+ when freely distributed in both the apparent and trapped supernatant and is obtained from the following equation.

$$K_f = \frac{(P.V.)_T \times K_s}{100}$$

K_s = conc. of K. as measured in the supernatant

$(P.V.)_T$ = The true plasmatocrit and equals

$$= (P.V.)_{app} + 13 \frac{Hct}{100}$$

$(P.V.)_{app}$ = 100 - haematocrit (Hct)

Hct in this experiment represents the percentage packed cell volume as determined after centrifugation of 10 ml blood in 10 ml capacity conical TPX centrifuge tube for 20 minutes at 3000 rpm in MSE Super-Minor Centrifuge.

The number (13) refers to the standard percentage of trapped plasma (when 10 ml blood are centrifuged in 10 ml conical TPX centrifuge tube for 20 minutes at 3000 rpm in MSE Super-Minor centrifuge).

Determination of the percentage of trapped plasma:

Into a 10-ml conical TPX centrifuge tube 5 ml of previously frozen washed erythrocytes were placed. To the latter 5 ml of isotonic saline were added, followed by two drops of dilute Blue Dextran-2000 dye solution in 0.9 percent saline.

After thorough mixing the tube was centrifuged in MSE Super-Minor centrifuge at 3000 rpm for 20 minutes. The supernatant volume was measured, aspirated by a Pasteur pipette and collected in a dry clean tube and marked supernatant (1).

The cells were resuspended to the original volume by isotonic saline, mixed and recentrifuged for 20 minutes at 3000 rpm. The supernatant solution was removed and marked supernatant (2). The optical density (O.D.) of both supernatant were measured in an EEL-SPECTRA colorimeter at 650 nm using 10 mm light path cuvette. A solution of saline is used as a blank. Previous experience had shown that haemoglobin did not interfere with Blue Dextran dye at that wave length. The percentage of trapped plasma was calculated from the following formula:

$$\text{percentage of trapped plasma} = \frac{\frac{V_2 \times S_2}{S_1 - S_2}}{\text{P.C.V.}} \times 100$$

where

V_2 = volume of the second supernatant

S_1 = O.D. of the first supernatant

S_2 = O.D. of the second supernatant

P.C.V. = volume of the packed cells

The experiment was repeated several times and the answer was always the same. The percentage of trapped plasma was 13%.

ALUMINIUM ESTIMATION

At least 100 ml of previously frozen thawed and washed erythrocytes contained in the processing bag were sent to the Department of Health and Social Security (D.H.S.S.) for estimation of the aluminium level

in the blood. Aluminium was estimated by the atomic absorption spectroscopic technique.

Blood pH:

This was measured at 20°C with a Corning - EEC Model 110 digital pH meter.

HAEMATOLOGICAL MEASUREMENTS

The red cell count, white cell count, mean cell volume, haematocrit, mean cell haemoglobin, mean cell haemoglobin concentration were determined in a Coulter Counter Model "S".

CALCULATION OF RED CELL RECOVERY

Calculation of the percentage of red cell recovery was based on the sum of supernatant haemoglobin in all the wash solutions plus the intact cellular haemoglobin in the resuspended cells. This was assumed as 100% value and rarely agreed with the total haemoglobin input into the can. The reason is obscure but may be related to the technical problems of estimating accurately the haemoglobin dissolved in different solutions before and after processing.

$$\text{percentage red cell recovery} = \frac{\text{intact cellular Hb recovered}}{\text{intact cellular Hb recovered + sum of Hb in wash solution.}} \times 100$$

BACTERIOLOGICAL EXAMINATION

One hundred units of packed red cells were used in this study. All the units were obtained by random from the routine red cell concentrate stocks which have been stored for five days or less. The packed red cells were individually glycerolized, frozen and stored in the vapour phase of liquid nitrogen, as described previously, for variable periods of time. On the day of processing the desired unit was taken out of the refrigerator, thawed at 40°C and processed in the IBM 2991 Automatic Cell Processor. At the end of the washing procedure the red cells were resuspended in approximately equal volume of isotonic saline that contained 70 ml of acid-citrate-dextrose solution. While the red-cell suspension was mixed, by reciprocal agitation of the centrifuge bowls, the line used for introduction of the suspension medium was modified so that sampling from the bag under sterile conditions was possible. This was achieved by attaching, to the plastic spear, a short sterile plastic junction, which terminated in a male luer fitting, into the latter a sterile disposable needle 18G was fixed.

A sample of 5 ml of the washed resuspended cells was then pumped into each of the following containers:

a. Bacto-Blood culture bottle containing 50 ml of Fluid Thioglycolate medium (Difco laboratories) for anaerabic culture at 37°C.

b. Bacto-Blood culture bottle containing 50 ml Fluid Tryptic Soy Broth (Difco laboratories) for aerobic culture at 37°C.

c. Bacto-Blood culture bottle containing 50 ml Fluid Tryptic Soy Broth for aerobic culture at 20°C.

The above bottles served as primary cultures and were maintained at the specified temperature for seven days.

When all the samples required for examination were obtained the tubing harness and the rotating seal were cut off and the bag containing the resuspended cells was kept in a conventional refrigerator at $+4^{\circ}C$.

On the seventh day of incubation of the primary cultures, subcultures were performed onto blood agar plates (Oxoid). Three plates, corresponding to the three primary culture bottles, were used for each unit. The blood plates were incubated as follows:

One plate kept aerobically at $20^{\circ}C$ (room temperature)
A second plate was maintained aerobically in an incubator at $37^{\circ}C$. The third plate was incubated at $37^{\circ}C$ inside self containing gas pack Anaerobic System (a transparent polycarbonate jar in which an anaerobic atmosphere was generated with the aid of specially designed foil envelope that generates hydrogen + carbon dioxide). An anaerobic indicator should always be used to make sure that the system is working perfectly. All plates were examined after 72 hours. If bacterial growth was present, a 1.0 ml aliquot was obtained from the original bag and a colony count was performed on nutrient agar. At the same time samples were taken for repeated cultures.

Since one of the main objects of this study was to extend the shelf-life of the processed blood from a few hours to several days, examination for bacterial contamination after storage was essential. For this reason samples from the original units were aseptically withdrawn after 10 days of post-thaw storage, these were innoculated onto bottles of fluid Thioglycolate and Tryptic Soy Broth and examined in the same way as those obtained on zero day of processing.

2,3 DIPHOSPHOGLYCERATE (2,3 DPG) DETERMINATION

Principle:

In 1970 Rose and Liebowitz found that the enzyme phosphoglycerate mutase (PGM), prepared from rabbit's mucles, possesses a phosphatase activity which is capable of hydrolizing 2,3 DPG into 3 - phospho-glycerate (3-PGA) and phosphate.

$$2,3\ \text{DPG} \xrightarrow[\text{Glycolate - 2 - phosphoric acid}]{\text{2,3 DPG phosphatase}} 3 - \text{PGA} + \text{P}$$

The enzymatic activity which catalyzes this reaction was called 2,3 DPG phosphatase and it was necessary to add Glycolate 2 - phosphoric acid (Phosphoglycolic acid) as a stimulant for the reaction. One phosphate group is produced from the hydrolysis of one mole of 2,3 DPG. This released phosphorus could be measured colorimetrically at 660 nm by means of the Fiske and SubbaRow reaction. This formed the basis of a procedure for the determination of 2,3 DPG in the blood in this study.

Procedure:

All the reagents for this procedure were supplied by Sigma Co. (Kit No. 665). Before starting to measure the 2,3 DPG in the test samples a calibration curve was first prepared as follows:-

1. Into six test tubes marked 1,2,3 .....6, the following reagents were added:

Tube No.	Phosphorus standard (20 µg/1 ml) (Stock 661-9) ml	Distilled water ml
1	0	5.1
2	0.3	4.8
3	0.6	4.5
4	0.9	4.2
5	1.2	3.9
6	1.5	3.6

2. To each tube add 1.0 ml of acid molybdate solution (Reagent B) and contents are gently mixed by shaking.

3. Add to each tube 0.25 ml of the Fiske and SubbaRow reducer solution (Reagent F). The contents are then mixed by inversion and allowed to stand at room temperature for 10 minutes for the colour to develop.

4. Transfer to 10 mm lightpath cuvette and read the optical density (O.D.) of all tubes at 660 nm using tube No.1 as a reference. Reading was carried out in EEL-Spectra-colorimeter and has to be completed within an additional 10 minutes (total 20 minutes from the time the Fiske and SubbaRow reagent was added).

Tube No.	The Optical Density obtained	Microgram phosphorus per tube	µ Moles of 2,3DPG 1 ml of blood
1	0	0	0
2	0.11	6	1.55
3	0.22	12	3.10
4	0.33	18	4.65
5	0.44	24	6.2
6	0.56	30	7.75

The curve obtained by drawing the 2,3 DPG content per ml whole blood against the optical density at 660 nm was a straight line. All measurements in this study were performed on the same colorimeter and the results were read from the same calibration curve.

Procedure for 2,3 DPG Determination

(Sigma Tentative Technical Bulletin No.665 1970)

1. One ml of the blood sample (packed cells or whole blood) is pipetted into a test tube containing 3 ml of cold 8% Trichloroacetic acid. The mixture is vigorously shaked for several seconds after which it is centrifuged for 10 minutes at 3000 rpm (in MSE Super-Minor Centrifuge) to obtain a clear protein free supernatant.

2. Two test tubes are prepared and labelled Blank and Test, into which the following reagents were added (mix after each addition by swirling)

	Blank	Test
Protein free supernatant from step (1)	0.5	0.5
Triethanolamine buffer (Stock No.665-5)	2.5	2.5
Phosphoglycerate mutase (Stock No.665-3)	0.02	0.02
Phosphoglycolic Acid Solution (Reagent C) Stock No.665-2	-	0.1 ml

3. Both tubes are then incubated in a 37°C water bath for 15 minutes for the reaction to take place.

4. Remove from the water bath and add to BOTH tubes 2.0 ml 8% trichloroacetic acid (Stock No. 665-8) to stop the reaction. The contents are mixed by inversion.

5. To the BLANK tube only, add 0.1 ml phosphoglycolic acid solution.

6. To each tube add:

1.0 ml Acid molybdate solution Stock No. 661-11

0.25 ml Fiske and SubbaRow reducer solution (Stock No. 661-8)

The contents are then mixed by inversion and left for 10 minutes at room temperature for the colour to develop.

7. Transfer to 10 mm lightpath cuvettes and read the O.D. of the test tube at 660 nm using the blank as a reference. This reading is then used to determine the 2,3 DPG concentration in one ml sample from the calibration curve.

8. The haemoglobin concentration in the test blood was determined by the cyanemethaemoglobin method and the 2,3 DPG concentration is related to one gram haemoglobin according to the following formula:

concentration of 2,3 DPG per gram haemoglobin =

$$\frac{\text{conc. of 2,3 DPG/ml sample}}{\text{conc. of Hb/ml of the sample}}$$

Reagents for 2,3 DPG estimation

1. Triethanolamine Buffer Solution

Contains Magnesium ions and EDTA obtained from Sigma London Chemical Co. Ltd. Stock No. 665-5.

2. Phosphoglycerate Mutase (PGM)

Enzyme suspension in Ammonium sulfate obtained from Sigma London Chemical Co. Ltd. Stock No. 665-3.

3. Phosphoglycolic acid

50 mg preweighed vial reconstituted with 5.0 ml distilled water. Obtained from Sigma London Chemical Co. Ltd. Stock No. 665-2.

4. Acid molybdate solution

contains:

$$1.25\ (NH4)_6\ MO_7\ O_{24}\cdot\ 4\ H_2O \text{ in } 2.5\ H_2\ SO_4$$

Obtained from Sigma London Chemical Co. Ltd. Stock No. 661-11.

5. Fiske and SubbaRow reducer

Supplied by Sigma London Chemical Co. Ltd. as 1.0g solid in vials. The contents of the vial are dissolved in 6.3 ml distilled water. Stock No. 661-8.

6. Trichloroacetic acid 8%

Prepared locally by dissolving 8g of Trichloroacetic acid (M&B) in 100 ml of distilled water.

ADENOSINE TRIPHOSPHATE (ATP) DETERMINATION

Principle:

One phosphate radicle is removed from ATP and transferred to 3- phosphoglycerate (PGA), to form 1,3 diphosphoglycerate. This reaction is catalyzed by the enzyme phosphoglyceric phosphokinase (PGK).

$$\text{ATP} + \text{3-phosphoglycerate} \xrightarrow{\text{PGK}} \text{ADP} + \text{1,3 diphosphoglycerate.}$$

Another enzyme glyceraldehyde phosphate dehydrogenase (GAPD) is used to transfer the 1,3 diphosphyglycerate into glyceraldehyde-3-phosphate. Reduced coenzyme I (diphosphopyridine nucleotide (DPNH) acts as hydrogen donor in this reaction, and as a result it is oxidized to DPN.

$$\text{1,3 diphosphoglycerate} + \text{DPNH} \xrightarrow{\text{GAPD}} \text{glyceraldehyde-3-phosphate} + \text{DPN} + \text{P}$$

As a result of oxidation of DPNH to DPN the optical density at 340 nm is decreased, and this forms the basis for ultraviolet determination of ATP.

Procedure:

The first step in this determination is the formation of a protein-free filtrate from the blood by precipitation of the protein by 0.6 M perchloric acid. On application of the method of preparation of protein free filterate from frozen blood as recommended by Sigma Chemical Company (the supplier of the Kit) we found great difficulty in obtaining a clear supernatant. A slight modification was introduced at this stage for the precipitation of the proteins and separation of a clear supernatant.

1. One ml of blood is added to 1.0 ml of 0.6 M perchloric acid in a conical TPX centrifuge tube.(The contents of the tube are immediately mixed on a whirli-mixer for several seconds. The mixture is then kept in a refrigerator for an additional 15 minutes to insure complete protein precipitation).

2. Centrifuge for 10 minutes at 3000 rpm to obtain as clear a supernatant as possible.

3. Using a pasteur pipette the supernatant is aspirated and collected in 2 ml - capacity polycarbonate microcentrifuge tubes which are then spun in a microcentrifuge (Quickfit-Instrumentation) for one minute at 12,000 rpm. The resulting supernatant was always clear.

4. Into a 0.3 mg - DPNH vial, No. 340-13 the following reagents are added:

1.0 ml PGA buffered solution stock No. 366-1

1.8 ml distilled water

0.2 ml of the clear supernatant prepared in step (3).

The vial is then capped and the contents dissolved by inversion.

5. The content of the vial is transferred into a cuvette of 10 mm lightpath making sure to pour as much of the contents as possible into the cuvette.

6. The optical density is read at 340 nm (in the ultraviolet range) in Uvichem Spectrophotometer. This reading is recorded as the INITIAL O.D.

7. With the aid of Hamilton Dispensing Syringe 0.04 ml GAPD/PGK (Stock No. 366-2) is added to the cuvette which is then inverted gently for several times and replaced again in the spectrophotometer and left for at least 5 minutes before the FINAL O.D. is recorded.

Calculation:

$$\Delta \text{ OD} = \text{INITIAL OD} - \text{FINAL OD}$$

$$\mu \text{ Moles of ATP per ml} = \frac{\Delta \text{ OD}}{(6.22)} \times 3.0 \times 10$$

where

The factor (3.0) represents the volume of liquid in the cuvette.

The factor (6.22) is the millimolar extinction coefficient for β-DPNH at 340 nm. Since the reaction mixture contains the equivalent of 0.1 ml of original sample, the factor 10 is used to correct it to 1 ml.

The haemoglobin concentration of the blood under investigation is determined by the cyanemethaemoglobin technique. The ATP concentration is expressed as μ Moles per gram haemoglobin using the following formula.

$$\mu \text{ Moles ATP/gram Hb} = \frac{\mu \text{ Moles ATP per ml}}{\text{Hb concentration gram/ml}}$$

Reagents for the Ultraviolet Determination of

ADENOSINE - 5' - TRIPHOSPHATE

Kit No. 366

Used for 30 assays and contains

a. B - DPNH Stock No. 340-13

30 Preweighed vials, each vial contains 0.3 mg β -DPNH.

b. PGA Buffered Solution, Stock No. 366-1 50 ml bottle.

3-Phosphoglyceric acid containing magnesium ions and triethanolamine at pH 7.8 at 25°C. Stable for six months when stored at 0-5°C.

c. GAPD/PGK Enzymes, Stock No. 366-2

1.5 ml vial containing glyceraldehyde phosphate dehydrogenase and phosphoglyceric phosphokinase suspension in ammonium sulfate. Stable for six months when stored at 0-5°C.

d. 0.6 M Perchloric acid solution

This item does not come with the previous kit and is prepared locally in the laboratory as follows:

Pipette 5.0 ml perchloric acid 70% and

dilute to 100 ml with distilled water,

this gives a0.6M solution.

The kit 366 was supplied by Sigma London Chemical Co. Ltd.

12 Lettice Street, London S.W.6. England.

ESTIMATION OF RESIDUAL GLYCEROL AND SORBITOL CONTENT IN THE FROZEN BLOOD

Glycerol and sorbitol content in the supernatants of the previously frozen processed red blood cells was estimated by gas-chromatography of their respective alditol acetate (Niedermeier 1971), using mannitol as a reference.

Principle:

When acetic anhydride is added to either glycerol or sorbitol a volatile Tri-O-acetyl glycerol or Hexa-O-acetyl sorbitol is produced. These substances could then be estimated by gas-chromatography with reference to a substance of known concentration e.g. mannitol which is similarly treated. Pyridine is added with the acetic anhydride to prevent any increase in the hydrogen ion concentration that results from the formation of acetic acid as a by-product of the reaction.

Method:

1. The following standards were prepared:

STANDARD I : a solution of mannitol in water containing 5 µg/µl

STANDARD II : a mixture of glycerol, mannitol and sorbitol (2.9 µg/µl of each in water).

2. The test samples are prepared from the supernatant fluid of previously frozen-processed red blood cells before the addition of any resuspending medium.

3. 1.0 ml volume of the test sample was pipetted into Amicon (F.50) ultrafilteration cone which was fitted into an MSE 50 ml centrifuge tube.

4. The sample was ultrafiltered by spinning in an MSE Super-Minor centrifuge for 5 minutes at 3000 rpm.

5. A volume of 25 µl of the filtrate was added to an equal volume of STANDARD I (mannitol) in a Kontes 0.3 ml Microflex vial which was then marked TEST.

6. 50 µl of a STANDARD II Solution was pipetted into another Kontes 0.3 ml vial which was marked STANDARD.

7. The contents of both vials were lyophilized.

8. Into both vials the following reagents were added to the lyophilized materials:

   50 µl acetic anhydride (BDH - Analar)

   25 µl pyridine (BDH - Analar)

9. The vials were then placed in Statim Thermoblock preheated to 100°C for 60 minutes. 1.5 µl of the content of the STANDARD vial was then rapidly injected through the rubber septum into a gas-chromatography colomn (Hewlett Packard Model 402 FID). The apparatus was preheated to 115°C and programmed so as to increase by 5°C every

minute to a final temperature of 200°C. The colomn which is 4mm x 2.0 meters was packed with 3% ECNSS-M on AW-HMDS gas chrom Q 100/120 mesh.

11. The compounds contained in STANDARD II solution appeared in the following sequence:

Glycerol after 2.6 minutes

Mannitol after 18.8 minutes

Sorbitol after 20 minutes

The appearance of these compounds was recorded on a chart in the form of triangular areas. The surface area of these triangles was recorded with a Vilar 6230 Electronic Integrator.

12. When the elution of all the constituents of the STANDARD II was completed, the apparatus was allowed to cool and the same process was then repeated with the TEST samples.

Calculation:

glycerol content of the test sample gram/100 ml =

$$\frac{\frac{G}{M} \times 5}{(R.F.)_G \times 1000} \times 100$$

Sorbitol content of the test sample gram/100 ml =

$$\frac{\frac{S}{M} \times 5}{(R.F.)_S \times 1000} \times 100$$

G = The surface area of the glycerol triangle in the test sample

M = The surface area of the mannitol triangle in the test sample

S = The surface area of the sorbitol triangle in the test sample

$(R.F)_G$ = The response factor for glycerol and =

$$\frac{\text{The surface area of the glycerol triangle in the STANDARD sample}}{\text{The surface area of the mannitol triangle in the STANDARD sample}}$$

$(R.F)_S$ = The response factor for sorbitol and =

$$\frac{\text{The surface area of the sorbitol triangle in the STANDARD sample}}{\text{The surface area of the mannitol in the STANDARD sample}}$$

ESTIMATION OF 24-HOUR POST-TRANSFUSION SURVIVAL OF AUTOLOGOUS FROZEN BLOOD

From each volunteer, usually a member of the staff, 400 ml of blood were collected into plastic packs containing 70 ml ACD or CPD whichever is available. The pack was centrifuged at 1,300g for 60 minutes and the plasma separated. The packed red cells retained in the plastic pack were glycerolized, frozen and stored in the vapour phase of liquid nitrogen at -180°C. When required the can was taken out of the refrigerator and thawed as described previously.

Chromium labelling:

Approximately 30 µCi of ^{51}Cr as sodium chromate (Radiochemical Centre Amersham) was injected into the can immediately post-thaw and prior to washing, through the rubber Subaseal, under strict aseptic precautions. The can was immediately agitated and kept on an automatic Coulter Mixer for 30 minutes at room temperature.

Washing:

When manual washing was desired, the contents of the can were transferred into a Tuta five tail washing pack and processing was proceeded according to the method described earlier. In the IBM 2991 Automatic Cell Processor the cells were washed according to the programme described in the previous chapter.

Post-thaw Storage:

Units processed by the manual technique were transfused to the original donor after resuspension in isotonic saline on the same day and immediately after processing. Some of the units processed by the

IBM 2991 were transfused on the day of processing resuspended in saline, others were resuspended in 70 ml of acid-citrate dextrose solution and stored for up to five days at +4°C before being autotransfused. In each case the bag containing the blood to be transfused was weighed immediately before transfusion.

Infusion and sampling:

The subjects were rested for at least 15 minutes prior to transfusion to avoid any fluctuations in the packed cell volume which may be caused by muscular exercise or excessive movements.

2. A sample of 10 ml venous blood was withdrawn from the cubital vein. This blood was collected into a lithuim-heparin tube (Searle) and used for the following estimations:

a. Haematocrit

b. Haemoglobin content of the blood

c. Plasma haemoglobin

d. Other haematological parameters

3. The gross weight of a giving set as well as the blood pack was determined to one tenth of a gram on a top-pan balance.

4. The whole unit was infused, saving about 12 ml in the recipient line, into the subject within approximately five minutes through 15G needle or through 16G Medicut cannula (Aloe-Medical).
The time of start and finish was recorded.

5. Three 12 ml samples were withdrawn from the opposite arm at 15,30 and 45 minutes, of these, 2.5 ml was collected into an EDTA tube for estimation of haematocrit and other haematological parameters and the rest was collected on lithuim heparin.

The latter samples were used for:

a. Triplicate one ml samples of whole blood placed in Tek lab tubes for radioactive ^{51}Cr counting.

b. The rest is centrifuged and plasma separated and distributed into three 1.0 ml aliquots in Tek-lab tubes for radioactive iodine counting and another sample for estimation of plasma haemoglobin.

6. The emptied pack and giving set were reweighed and this weight was subtracted from the pretransfusion gross weight to obtain the net weight of the infused blood. This when divided by the density will give the volume of the blood transfused:

About 12 ml sample was removed from the giving set for:

a. Haematocrit and other haematological parameters estimation, 2.5 ml in an EDTA tube.

b. Estimation of density on Anton Parr - DMA 10 - Digital Densitimeter, (about 2 ml).

c. Radioactive counting - Three 1.0 ml aliquots in Tek-lab tubes.

d. Separation of supernatant from the remaining blood after centrifugation for 10 minutes at 3000 rpm. Free haemoglobin level was determined in this supernatant by the method described previously.

7. Another sample of 12 ml venous blood was withdrawn from the recipient after 24 hours for ^{51}Cr count. When determination of the life-span of the transfused cells was required additional samples were withdrawn every week for four successive weeks.

Determination of plasma volume:

Human albumin, labelled with ^{125}I, was used for these estimations. This was either injected separately into the recipient or given simultaneously with the chromium labelled cells by injecting the ^{125}I - labelled albumin into the blood pack immediately before transfusion. Both methods will be described in detail.

1. Method using separate injection of ^{125}I - labelled human serum albumin:

In the first five experiments of this study, those units processed by the manual technique, the material used for injection was prepared sterile and supplied by the University Department of Medical Physics as 5 μCi ^{125}I in 12 ml aliquots.

a. About 10 ml of this solution was taken into a sterile syringe, and all the bubbles removed. The syringe with the needle and its guard were accurately weighed.

b. The contents of the syringe were then injected intravenously into one of the cubital veins. The empty syringe was reweighed to obtain the weight of the injected solution. One gram of the solution was considered equal to one ml without substitution of the density.

c. Blood samples, of about 12 ml volume, were collected at 15, 30 and 45 minutes from the opposite arm to avoid contamination from the injection site. These samples were dealt with as described above.

d. Radioactive iodine standards were prepared by measuring 1.0 ml of the labelled solution and transferring it to 100 - ml graduated flask already containing about 5 ml of 10 per cent solution of carrier unlabelled human albumin. The contents

of the flask were then diluted to exactly 100 ml with distilled water containing 1g/litre potassium iodide. Triplicate 1.0 ml aliquots from this standard were placed in Tek-lab tubes to be counted with the samples.

2. Method using simultaneous injection of ^{125}I labelled albumin and ^{51}Cr-labelled red blood cells:

In the remaining experiments of this study the ^{125}I-labelled human albumin was supplied as 1.0 ml iodinated (^{125}I) human albumin (from the Radio chemical Centre Amersham) containing 5 μ Ci of radioactive ^{125}I. The volume injected, varied according to the time of storage of the solution. In all the experiments we used the volume that contained 5 μCi of radioactive ^{125}I. The required volume was injected into the blood pack immediately before transfusion and the contents were thoroughly mixed. In this case the ^{125}I standard was the whole blood as collected from the recipient set.

Radioactive counting:

The activity of all samples was counted in well-type-scintillator gamma counter (Tracerlab-Gamma/Guard 150). The radioactivity was measured in triplicate aliquots of one ml of both the standard and samples. Counting was performed for a minimum of 5 minutes. With the method using single injection, since some of the ^{51}Cr gamma radiation would be detected under the optimum conditions for ^{125}I, a pure ^{51}Cr preparation was also measured under optimum conditions of both ^{125}I and ^{51}Cr. The percentage of ^{51}Cr contribution in the ^{125}I counts was determined and the actual ^{125}I activity was calculated.

CALCULATION

A. Calculation Of Plasma Volume:

1. When ^{125}I-labelled serum albumin was administered separately:

$$PV = \frac{V\ D\ Ns}{N_o} \text{ or } \frac{V\ D\ Ns}{N_{15}f} \text{ or } \frac{V\ D\ Ns}{\frac{N_{15}+N_{30}+N_{45}}{3}}$$

PV = Plasma volume in ml.

V = Volume of labelled albumin solution injected

D = Dilution factor of the standard

N_s = Average ^{125}I activity of the standard in CPM/ml (Count per minute per ml).

N_o = ^{125}I activity of the plasma sample corrected to zero time.

2. When ^{125}I - labelled albumin solution was simultaneously introduced with the ^{51}Cr-labelled red cells

In this case the ^{125}I counts must first be corrected for the ^{51}Cr activity interference. This was calculated according to the following formula:

$$I' = I - C \times \frac{S_1}{S_c}$$

I' = The corrected ^{125}I sample value (CPM)/ml

I = Uncorrected ^{125}I sample value (CPM)/ml

C = The ^{51}Cr sample value (CPM)/ml

S_1 = Activity of pure ^{51}Cr preparation as measured in the ^{125}I channel (CPM/ml).

S_c = Activity of pure ^{51}Cr preparation as measured in the ^{51}Cr channel (CPM/ml).

Plasma volume was then calculated according to the formula:

$$PV = \frac{Ns\ V}{No} \quad \text{or} \quad \frac{V\ Ns}{N_{15} f} \quad \text{or} \quad \frac{V\ Ns}{\frac{N_{15} + N_{30} + N_{45}}{3}}$$

PV = plasma volume (ml)

N_s = Average ^{125}I activity of the transfused blood (after correction for ^{51}Cr interference) CPM/ml.

V = Volume of transfused blood (ml)

f = (1.02) and represent the rate of disappearance of ^{125}I from the circulation in 15 minutes

N_o = ^{125}I activity of the plasma sample corrected to zero time (CPM/ml)

B. Calculation of the Blood Volume:

1. $$WBV = \frac{PV}{1 - \frac{0.9\ (H_{15} + H_{30} + H_{45}}{3}}$$

WBV = Whole blood volume (ml)

PV = estimated plasma volume

0.9 = Correction factor = $\frac{\text{whole body haematocrit}}{\text{peripheral venous haematocrit}}$

H_{15} = Venous haematocrit at 15 minutes post-transfusion

H_{30} = Venous haematocrit at 30 minutes post-transfusion

H_{45} = Venous haematocrit at 45 minutes post-transfusion

2. Calculation of the blood volume using the ^{125}I counts:

$$WBV = \frac{N_s\ V}{C_o \times 1.05} \quad \text{or} \quad \frac{N_s\ V}{\frac{(C_{15} + C_{30} + C_{45})1.05}{3}}$$

WBV = Whole blood volume (ml)

N_s = Average ^{125}I activity of the transfused blood (CPM/ml)

V = Volume of blood transfused

C_o = ^{125}I activity of whole blood sample corrected to zero time

1.05 = Correction factor = $\frac{\text{Body plasmacrit}}{\text{Venous plasmacrit}}$

C. Calculation of the Red Cell Volume

$$RCV = WBV - PV$$

RCV = Red cell volume in ml.

WBV = Whole blood volume in ml.

PV = Estimated plasma volume (ml)

D. Calculation of the 100 per cent activity

$$A_{100} = \frac{C_s \; V}{RCV}$$

C_s = Average ^{51}Cr activity in the transfused blood (CPM/ml)

V = Volume of the blood transfused (ml)

RCV = Red cell volume (ml)

E. Calculation of the 24-hour Post-transfusion Survival

$$\text{24-hour post-transfusion survival} = \frac{A_{24}}{A_{100}} \times 100$$

$$A_{24} = \frac{^{51}Cr \text{ CPM/ml of whole blood sample}}{\text{Haematocrit}} \times 100$$

PREPARATION OF LYMPHOCYTE SUSPENSIONS

A. Preparation of Lymphocyte Suspensions From Small Volumes of Fresh Blood:

In this study pure lymphocytes suspensions were prepared from defibrinated blood. Peripheral venous blood was always used for this purpose.

Method:

1. Approximately 20 ml peripheral venous blood was collected into MacCartney bottles containing 15 x 4 mm glass beads.

2. Blood was defibrinated by gentle inversion of the tube for 10 minutes.

3. Six ml of defibrinated blood was carefully layered on top of 4.0 ml Ficoll Triosil (density gradient mixture) see page (196) in a 16 mm diameter plastic tube. Great care was taken to avoid mixing at the interface.

4. The tube was centrifuged for 25 minutes at 2000 rpm (400 x g). A thin white band of lymphocytes appeared at the interface between the serum above and the Ficoll-Triosil below, while the heavier erythrocytes and granulocytes sedimented to the bottom of the tube.

5. The lymphocyte band was harvested, using a Pasteur pipette, and collected into a sterile plastic tube (NUNC).

6. The lymphocytes were washed twice in large volume of 199 tissue culture medium. Each time the tube was spun for five minutes at 2000 rpm (400 x g).

7. After the last wash the lymphocyte-pellet was resuspended in 1.0

ml of 199 tissue culture medium. A drop of this suspension was transferred by Pasteur pipette, onto a Neubauer Haemocytometer Grid and a cell count performed under phase contrast microscopy.

The number of viable lymphocytes present in 5 small squares were counted. When this number was divided by 20 the number of lymphocytes (in millions) present in 1.0 ml suspension was obtained. Cells prepared by this technique were used for lymphocyte culture.

B. Preparation of Lymphocyte Suspensions from Large Volumes of Fresh Blood:

From a healthy volunteer 350 ml venous blood was collected into a half - MRC bottle that contained 250 glass beads. Blood was defibrinated by continuous gentle inversion for 10 minutes.

One volume (10 ml) of de-fibrinated whole blood was carefully layered on top of one volume Ficoll-Triosil in a plastic sterile universal tube. The tubes were spun for 15 minutes at 4000 rpm (2000g). The lymphocyte bands at the interface were havested and collected into 15 ml sterile plastic centrifuge tubes (NUNC).

The tubes were filled with 199 tissue culture medium and spun for 5 minutes at 2000 rpm (400g). The supernatant was decanted and replaced with fresh medium and the process was repeated once more. At the end of the second wash the lymphocyte pellet was resuspended in a 1 ml volume of 199 tissue culture medium and cell count performed as described above.

Lymphocytes prepared by this method were used after labelling with radioactive iodine ^{125}I for determination of the residual lymphocyte and lymphocyte debris in the frozen blood.

SEPARATION OF LEUCOCYTES FROM FROZEN BLOOD

My experience with the leucocyte content of the frozen blood has shown that only 5 per cent of the original leucocytes in the fresh blood remained intact after freezing, thawing and deglycerolization. The great majority (99%) of these leucocytes are lymphocyte in origin. So it was evident that any method to be applied for separation of leucocytes from the frozen blood is in fact a method for lymphocyte separation.

Some time was required to develop an efficient method for separation of lymphocytes from the frozen blood. At the beginning, attention was directed to a density gradient separation by a dextran solution that was successfully used by Chaplin et al (1959) for separation of leucocytes from fresh blood. A solution of 6 per cent dextran (M.W. 70,000) in 0.9 percent sodium chloride was tried in different volumes. However, I found that previously frozen-thawed-washed erythrocytes sedimented poorly in that solution. Dextran of higher molecular (250,000) and in various concentrations were also tried without success. Plasmagel and hydroxyethyl starch gave no better results than the dextran.

After exhaustive experiments I noticed that the addition of a small quantity of human plasma to the frozen-thawed-washed erythrocytes improved the sedimentation rate of the latter, in either dextran or hydroxyethyl starch, to a great extent. No satisfactory reason could be provided at this time to explain the beneficial effect of plasma in this respect, however it seems that the fibrinogen plays a major role in this phenomenon.

Because of the very low content of leucocytes in the frozen blood it was necessary to sacrifice the whole unit for separation of a reasonable amount of lymphocytes sufficient to perform one experiment.

The method routinely used for separation of lymphocytes in our laboratory employed the addition of small quantity of AB plasma to the erythrocytes after processing and was carried out as follows:

1. After thawing and processing in the IBM 2991 Automatic Cell Processor 40 ml of AB plasma was added to the erythrocytes in the processing bag. This was followed by the addition of 100 ml of 6 per cent hydroxyethyl starch (Fresenius-Pharma).
2. After thorough mixing the contents of the bag were dispensed as 30 ml volumes into 30 ml capacity sterile universal container "Sterlin Products".
3. The blood-HES-plasma mixture was then incubated for 45 minutes in 37°C incubator.
4. The supernatant fluid, containing the leucocytes, was then aspirated and collected into sterile centrifuge tubes (NUNC).
5. The tubes were spun for 15 minutes at 2000 rpm, (400g) the supernatant was decanted, replaced by 10 ml of 199 tissue culture medium and the process of washing was repeated once more.
6. At the end of the last wash the pellet was resuspended in sufficient volume of 199 tissue culture medium.
7. Because of red cell contamination, leucocytic count was performed by the visual method after lysing the red cells with a diluting fluid that contained 2 per cent acetic acid which was coloured violet with gentian violet dye.

PREPARATION OF PURE GRANULOCYTE SUSPENSION FROM LARGE VOLUMES OF FRESH BLOOD

1. Collection

400 ml of blood was collected by venepuncture from a healthy volunteer, into plastic pack containing 2250 U.S.P. heparin solution "Fenwal Laboratories".

2. Filtration

Granulocytes were separated from the blood by passing it through a Nylon-Leucocyte-filter, (Leuco-Pak) "Fenwal Laboratories", immediately after collection. Filtration was performed by placing the unit in a 37°C incubator. Granulocytes were retained on the filter as they adhere to the nylon material by phagocytosis. This property is enhanced at 37°C and at a slightly alkaline pH (7.4).

3. Elution

The adherent granulocytes were eluted from the filter by washing them with 400 ml ACD plasma pH 6.5. Plasma was passed through the filter under pressure of 250 m.m.Hg. The eluent was received into 500 ml sterile plastic transfer pack.

4. Separation

The granulocytes were sedimented from the suspension by centrifugation for 20 minutes at 500 rpm in a DAMON/-6 centrifuge, the temperature of which was adjusted to 10°C. This was followed by squeezing out of the supernatant fluid.

5. Purification

As the sedimented granulocytes were heavily contaminated with red

cells purification was achieved by sedimenting the red cells through a hydroxyethyl starch solution. 30 ml volume of 6 per cent hydroxyethyl starch solution was added to the cellular sediment in the transfer bag and after thorough mixing the contents were transferred to a 100 ml capacity measuring glass cylinder. The latter was incubated for 45 minutes at 37°C., after which period the supernatant fluid containing the granulocytes was aspirated and transferred to sterile plastic centrifuge tubes (NUNC). The tubes were spun for 15 minutes at 3000 rpm. the supernatant decanted and the cellular pellets resuspended in 2 ml "Shock Solution" (See below), thoroughly mixed on Whirlimixer and an additional 8 ml of "Shock Solution" was added. The content was mixed by gentle inversion of the tubes and then incubated for 10 minutes in 37°C waterbath.

After incubation the tubes were spun at 1000 rpm for 7 minutes, supernatant decanted and the cellular pellet resuspended in sufficient volume of 199 tissue culture medium.

Leucocyte count performed on a sample of the suspension as described previously.

Contents of the "Shock Solution"

Ammonium chloride 0.8%

Tri-Sodium EDTA 0.1%

Potassium dihydrogen phosphate 0.01%

PREPARATION OF PURE PLATELET SUSPENSION

1. Freshly prepared platelet concentrates were supplied by the Component Laboratory of our department in 20 ml. volumes.
2. The suspension was transferred into 50 ml "Oak Ridge" tube.
3. The platelet rich plasma was centrifuged for 20 seconds at 5000 rpm in MSE Super-Minor centrifuge.
4. Supernatant containing the platelets was aspirated and the volume replaced by isotonic saline and recentrifuged for another 20 seconds at 5000 rpm.
5. All the supernatant fluids were added together and spun for 10 minutes at 4000 x g.
6. The supernatant was then decanted and the cellular sediment was resuspended in 50 ml volume of isotonic saline.
7. The process of spinning and decantation was repeated once more after which the platelets were labelled with radioactive ^{125}I as it will be described later.

LABELLING OF THE CELL-MEMBRANE OF LEUCOCYTES OR PLATELETS WITH RADIOACTIVE (^{125}I - NaI).

This process was performed for the purpose of studying the leucocyte and/or platelet derived material of the frozen blood. Lymphocytes, granulocytes and platelets were studied separately in this respect and will be explained later in detail.

Principle:

"Chloramine T" is an oxidising agent, it releases iodine from sodium iodide salt. The released iodine will react with the tyrosine or unsaturated lipid content of the cell membrane and is prevented from penetrating into the inside of the cell by the fact that it remains attached to the unsaturated double bond in the fat. However as an extra precaution the reaction is stopped at the level of the cell membrane by the addition of sodium sulphite, a reducing agent. When sodium iodide salt that was labelled with radioactive ^{125}I is used then the cell membrane of any cellular suspension would be radioactively labelled.

Method:

This method was designed to label cellular material of 50/µl packed volume, however when larger volumes of cells are to be used, the reagent volumes could be consequently doubled according to the volume of the cell suspension.

1. The cell suspension is hard packed by centrifugation for 10 minutes at 3000 rpm.
2. Decant the supernatant.
3. Susperd the cellular-pellet in equal volume (50/µl) of phosphate buffered saline pH 7.5.
4. Add 200/µCi of ^{125}I labelled NaI (Radiochemical Centre Amersham-England).
5. Add 10/µl (50/µg) of chloramine T. solution.
6. Mix thoroughly on a whirlimixer, wait for 10 seconds then stop the reaction by the addition of 200/µl (120/µg) of sodium sulphite ($Na_2 S_2 O_5$).

7. Add 1 ml (200 µg) of potassium iodide (KI).

8. The excess radicactive material is removed by thorough washing of the cells as follows:

   a. The suspension containing the labelled cells is carefully layered onto 5 ml of 10 per cent solution of bovine albumin (Miles Laboratories inc.) in 0.9% NaCl. (In case the labelled cells were platelets this solution was made up of 3% bovine albumin in 0.9% Sodium Chloride Solution).

   b. Centrifuge for 10 minutes at 2000 rpm (400g) (5000 rpm with the platelet). The cells will thus sediment at the bottom of the tube leaving the supernatant fluid, containing the excess radioactive material, clear.

   c. Aspirate the supernatant fluid with a Pasteur pipette from above downwards taking care not to disturb the cellular-pellet.

   d. Resuspended in 1.0 ml phosphate buffered saline and layer the suspension again on top of 5 ml 10 per cent albumin solution.

   e. The process of washing is repeated for at least 6 times after which radioactivity is checked in samples from all the wash solutions and the final cell suspension to make sure that all the excess radioactivity is washed out.

9. After the last wash the cell-pellet was resuspended in sufficient volume of phosphate buffered saline. A drop was transferred by Pasteur-pipette onto a Neubauer Haemocytometer Grid and examined under phase contrast microscopy for counting and to ensure that there was no contamination by other cells.

MEASUREMENTS OF LYMPHOCYTE, GRANULOCYTE AND PLATELET DERIVED MATERIALS IN FROZEN BLOOD

Total lymphocyte, granulocyte, and platelet derived materials were separately measured in previously frozen-thawed-washed erythrocyte suspension by radioactive labelling of a pure suspension of each of these cells separately which were added to the blood to be frozen.

Methods:

1. Pure suspensions of lymphocytes, granulocytes and platelets were prepared separately by methods described above.
2. Radioactive ^{125}I - Na I was used as a cell membrane marker.
3. A total of 31 units of packed red cells of less than five days old were used in this study. 13 units were injected with labelled lymphocyte suspensions so that each unit received the volume equivalent to 50 million labelled lymphocytes. 8 units were used for granulocyte studies - each unit injected with the 50 million labelled granulocytes. 10 units used for the platelet study.
4. After thorough mixing the units were glycerolized, frozen in liquid nitrogen and stored in the vapour phase of liquid nitrogen for at least 48 hours.
5. When required the units were thawed at 40°C and processed in the IBM 2991 Automatic Cell Processor. Some units were processed with the usual programme i.e. with the buffing coat intact. Some were processed with the buffy coat removed after the last wash step. This was achieved by manual overriding of the optical device for a few seconds so that the upper layer of the packed cells, believed to contain most of the white cells, was washed out with the

supernatant. Other units were processed with the buffy coat removed after each wash step.

6. In order to find out the system that gives the best removal of the white cells, the efficiency of filtration of the following system was examined.
   a. BR-10-Blood Administration Set (Baxter).
   b. Ultipor-blood transfusion filter (Pall Corporation, Biochemical Product).
   c. Swank Transfusion Filter (Extracorporeal Medical Specialities Inc.).

   Following the washing procedure, each of these filters was separately fitted into the blood processing bag, some of the washed cells were allowed to pass through and a sample was collected for radioactive counting.

7. The volume of the wash fluid was measured and sample taken for haemoglobin measurement and radioactive counting.

8. The volume of the blood after glycerolization, thawing and washing was determined from the net weight and density. Samples were also collected after each of those steps for haemoglobin measurements and radioactive counting.

9. Triplicate 1.0 ml aliquots were prepared from each sample and radioactive counting was performed for all the samples on the same time in a well-type scintillator gamma counter (Tracer Lab. Gamma/Guard 150) for at least five minutes.

Calculations:

$$\text{Red cell recovery} = \frac{\text{Intact cellular haemoglobin recovered}}{\text{Intact cellular haemoglobin recovered} + \text{haemoglobin loss in the wash solution.}}$$

* 100 per cent radioactivity =

CPM/ml of post-thaw sample X volume of the blood post-thaw

* radioactivity recovered =

CPM/ml of post-wash sample X volume of the blood post-wash

* radioactivity removed =

CPM/ml of wash solution sample X volume of the wash solutions.

* percentage of the radioactivity recovered =

$$\frac{\text{Radioactivity recovered}}{100\% \text{ radioactivity}} \times 100$$

STUDY OF THE VIABILITY AND ANTIGEN-STATUS OF THE LEUCOCYTES IN THE FROZEN BLOOD

In this study viability of the leucocytes was determined by two different methods:

I. Dye exclusion method

II. Phytohaemagglutinin (PHA) stimulation method.

I. DYE EXCLUSION TEST

Equal volumes of the isolated leucocyte suspension and 0.1% Trypan blue dye in saline were incubated at 37°C for 30 minutes.

A sample of the mixture was then examined on a Neubauer haemocytometer under phase microscopy.

The percentage of viable cells was determined by counting those cells which exclude the dye from a count of 100 leucocytes.

II. PHA STIMULATION

This measures the ability of the separated lymphocytes to respond to PHA in tissue culture. The latter is expressed as DNA radioactive-thymidine- uptake of a known number of cells.

Method:

1. Lymphocyte suspensions of either fresh or frozen blood were prepared as previously described.

2. The volume of a cell suspension equivalent to one million lymphocytes was pipetted into each of two 100 mm x 14 mm sterile screw cap, tissue culture tubes (NUNC) which were marked TEST and CONTROL.

3. To the TEST tube 6 µg of Welcome purified PHA solution was added and the volume was then made up to 4.0 ml with tissue culture media prepared as page 195 item (5). To the CONTROL tube only tissue culture media was added to make the volume 4.0 ml.

4. After thorough mixing Triplicate 1.0 ml aliquots were prepared from each tube, so that each of the TEST cultures contained 250,000 lymphocytes and 1.5 µg PHA per ml and the CONTROL cultures contained only 250,000 lymphocytes per ml.

5. The cultures were then incubated in a 37°C incubator for five days after which 1.0 µCi of tritiated Thyimidine TRA-61 (Radio Chemical Centre Amersham) was added to each culture and the tubes were reincubated for an additional four hours.

6. The content of each tube was then poured into a separate well of a Millipore Filter box containing a Whatman GF/C glass fibre filter and washed with 40 ml of phosphate buffered saline pH 7.2 (Oxoid).

7. Acid insoluble materials were precipitated onto the filter by the addition of 20 ml of 5 per cent trichloroacetic acid.

8. Lipid materials were then dissolved and the remaining red cell pigments were decolourized by washing with 40 ml of methylated spirit (M & B).

9. Filters containing the acid precipitate were transferred into plastic scintillation counting bottles (NEN) and dried by incubation in 50°C oven for 1 hour.

10. The tubes were then taken from the oven left for a while to cool and then 10 ml of liquid scintillation solution NE231 (Nuclear Enterprizes) were added.

11. The activity was measured in a well-type B - scintillation Counter ICN Tracerlab Corumatic 200 and expressed as count per million cell per minute.

12. After subtraction of the CONTROL counts from that of TEST, the response to PHA of both frozen and fresh blood from the same person collected at the same time could be compared.

LEUCOCYTE ANTIGEN CONTENT OF THE FROZEN BLOOD

The leucocyte antigen content of the frozen blood was determined by the ability of lymphocytes and leucocyte derived material, separated from that blood, to stimulate lymphocytes of a third party in one way mixed lymphocyte cultures. This was measured in terms of DNA-radioactive thymidine uptake of the responding cells and compared to that stimulated by lymphocytes separated from the fresh blood of the same person.

Method:

1. Collection of blood

400 ml of venous blood was collected from a healthy volunteer into a plastic blood giving pack containing 70 ml ACD. This was followed by the withdrawal of 15 ml venous blood into each of two McCartney bottles containing glass beads. The blood in the McCartney bottles was defibrinated by gentle inversion for 10 minutes.

2. Glycerolization and freezing

The pack containing the ACD-blood was spun for 60 minutes at 1,300 x g at 20°C. The plasma was separated and the pack cells were glycerolized, frozen by immersion in liquid nitrogen and stored in the vapour phase until required.

3. Preparation of lymphocyte suspensions

a. Fresh stimulating lymphocytes - see above

b. Frozen stimulating lymphocytes - see above

c. Third party responding lymphocytes: these were either prepared fresh or supplied from the frozen lymphocyte stock by the tissue-typing and transplantation laboratory in our department. When the latter type of responding cells were used, the same batch of cells were used in both the frozen and fresh blood experiments.

4. Preparation of mitomycin treated lymphocytes

In order to produce a one way mixed lymphocyte culture the stimulant cells must be treated with mitomycin to stop them from being stimulated by the other cells. This was achieved by the following

procedure:

(i) The volume of lymphocyte suspension equivalent to 6 x 10^6 lymphocytes was pipetted into a 10 ml conical centrifuge tube. This was made up to 10 ml with tissue culture medium prepared in Step (5).

(ii) 250 µg (25 µg/ml) mitomycin (Dale Pharmaceutical) suspension was added to the mixture and the contents were thoroughly mixed and placed in a 37°C water bath for 30 minutes.

(iii) The tube was then spun for 5 minutes at 2000 rpm (400g), supernatant decanted and replaced with a new solution of tissue culture medium.

(iv) The process of spinning, decantation, and resuspension was repeated twice after which the cell pellet was resuspended in 1.0 ml of tissue culture medium and a cell count performed.

5. Preparation of tissue culture medium for growing the lymphocytes

(i) The following reagents were added to 180 ml of sterile distilled water into a half MRC bottle:

- 4.0 ml of T.C. bicarbonate solution 10% (Difco).
- 20 ml T.C. medium 199 x 10 X (Difco Laboratories)
- 14 ml of HEPES buffer solution 30 mH (H&W).

(ii) 40 ml of this mixture was removed and replaced by equal volume of pooled heat-deactivated human serum.

Prepared as follows:

10 young non-transfused volunteers (5 group O, 4 group A and 1 group B) are bled (400 ml) into plain half - MRC bottle. Serum was separated - deactivated by heating at 56°C for 30 minutes, sterilized by filtration, and dispensed in 20 ml volumes in Universal plastic containers which are

stored at -40°C.

(iii) 2.0 ml of penicillin-streptimycin solution, containing 10,000 units of penicillin and 1000 µg streptomycin per ml, was then added to the mixture to keep it sterile.

6. Preparation of Ficoll-Triosil for lymphocyte separation

Solution A:

9% Ficoll: (M.W. 400,000 Pharmacia, Uppsala, Sweden) 90gms Ficoll are dissolved in 1000 ml distilled water (better at 37°C water bath).

Solution B:

33.9% Triosil: (Triosil 440, Myegaard & Co. Oslo, Norway).
200 ml Triosil 440 are made up to 445 ml with distilled water.
Mix 960 ml solution A with 400 ml solution B. Adjust specific gravity to 1.076 - 1.078 Distributed into Medical Flats (approximately 75 ml into each).
Autoclave for 10 minutes at 151 lb/inch Store in the dark at 4°C.

One way mixed lymphocyte culture and PHA stimulation

1. Lymphocyte suspensions, to be used as stimulating cells, either from fresh or frozen blood and those to be used as responding cells were prepared as described previously.
2. Tissue culture medium was prepared as described above.
3. Stimulating and responding cells were used in the ratio of 3:1 and triplicate cultures were made from each experiment.
4. The following system was followed for the same blood both before and after freezing.

a. PHA Stimulation of the Test cells.
One million test cells + 6 µg PHA (Test tube)
One million test cells alone (CONTROL tubes)

b. PHA Stimulation of the responding cells (to check their viability).
One million responding cells + 6 µg PHA (TEST tube)
One million responding cells alone (CONTROL tube)

c. One way mixed lymphocyte culture
3.0 million test cells (mitomycin treated)+ 1.0 million responding cells.

Each of these mixtures was prepared in 100 mm x 14 mm screw cap sterile tissue culture tubes. The volume of each tube was then made up to 4.0 ml with tissue culture medium.

5. After thorough mixing triplicate 1.0 ml volume cultures were prepared from each suspension and dispensed into new 100 mm x 14 mm tissue culture tubes so that each tube contained one fourth of the corresponding quantity.

6. The cultures were then incubated in a 37°C incubator for 5 days at the end of which 1 µCi of tritiated thymidine (TRA 61) was added to each tube and the tubes were then reincubated for an additional 4 hours.

7. Each culture was then washed onto Whatmann GF/C glass fibre filter in a Millipore filter box using phosphate buffered saline pH 7.2.

8. Following 40 ml saline wash, acid insoluble materials were precipitated on the filter by the addition of 20 ml of 5% trichloroaecetic acid.

9. Lipid materials were dissolved and the remaining red-cell pigments were decolourized by washing with 40 ml methylated spirit.

10. Filters containing the acid precipitate were then transferred into plastic scintillation counting bottles and dried at 50°C for at least one hour.

11. After cooling 10 ml of liquid scintillation solution NE231 were added and the activity measured in a well-type scintillation counter. (ICN Tracer lab Corumatic 200).

IN VIVO TESTING OF THE FROZEN BLOOD ANIGENICITY IN RABBITS

1. Rabbit injection:

15 Dutch White Rabbits were screened for the absence of antihuman lyphocytotoxic antibodies before the experiment begun.

The rabbits were divided into three groups

Group I (Nos. 1,2,3,4,15) received fresh blood

Group II (Nos. 5,6,7,8,9) (received frozen-swank filtered blood)

Group III (Nos. 10,11,12,13,14) (received frozen blood).

Each rabbit received 1.0 ml of blood, (50 per cent haematocrit) by ear vein on a fixed day of the week.

Before the blood was injected a 2.0 ml sample of venous blood was collected in a plain tube which was left in a refrigerator at +4°C overnight. Serum was separated by centrifugation and stored in a deep freeze until required.

The experiment was repeated at four weekly intervlas, a total of four injections and five bleedings.

The blood used on any one day was collected from a single donor. All donors used have the same ABO group and Rh type.

II. Preparation of Blood:

On the day before injection, 400 ml of venous blood was collected from a healthy volunteer into a plastic pack containing CPD anticoagulant. This was followed by the collection of 20 ml venous blood into a McCartney bottle containing glass beads for defibrination.

The blood was processed as follows:

Pure lymphocyte suspension was prepared from the defibrinated blood by separation on Ficoll-Triosil. The lymphocytes were washed twice and suspended in human 10% AB - serum, 80% McCoy's 5a medium, DMSO 10%. The lymphocyte suspension was then dispensed in straws each containing 0.5 ml (5 million lymphocytes).

10 ml CPD-blood was taken for injection into rabbits No. 1,2,3,4 and 15. The remaining cells were then packed and the plasma removed.

The packed cells were glycerolized and 20 ml of the mixture was taken and frozen separately in small aluminium tins according to the method described by Pepper et al (1973). On the day of injection (after 24 hours) the frozen unit was thawed, washed by the IBM 2991 Automatic Cell Processor and half of it was filtered through a Swank filter.

Both the filtered and unfiltered blood was diluted to 50% haematocrit with isotonic saline before injection into the corresponding rabbits.

Screening:

When four successive weeks had elapsed the post-immunization sera were adsorbed with a red cell pool from the small aliquots of that

frozen in the small tins to remove red cell antibodies. The pre-immunization samples together with the four adsorbed sera were screened simultaneously against a mixed pool of lymphocytes prepared from the straws that were previously frozen.

LYMPHOCYTOTOXICITY TEST

Each of the pre-immunization and adsorbed sera was serialy diluted, up to 1:512, with heat inactivated human AB- serum.

Sera were then plated out in one microlitre amounts, using a microdispenser (Hamilton), in a microtest plate (Home made) under liquid paraffin. Duplicate examination of each sample was performed. Lymphocyte pools from the four donors, which were previously frozen, were prepared by rapid thawing of the straws in an excess of warm 199 tissue culture medium (prepared as previously described). The lymphocytes were washed twice in the same medium and resuspended in AB heat inactivated human serum. The suspension was standardized to contain 8 million lymphocytes per ml.

One volume of lymphocyte suspension was diluted with three volumes of rabbit complement and 1.0 microlitre of the mixture was added to each well of the microtest plate (containing 1 µl of the rabbit sera). A positive control was set up with each plate consisting of 1.0 µl of anti-human lymphocyte globulin (supplied by the Tissue-Typing Laboratory). A negative control of heat inactivated AB-serum was also included. The plates were incubated for one hour at 37°C. One µl of 1.2% Trypan blue was added to each well and the test was read under a phase contrast microscope.

A positive test is that well in which 70% of the lymphocytes were found dead (i.e. had taken up the dye).

THE RESULTS

1 - Characterization of fresh and liquid stored blood

In order to standardize the methods used in the evaluation of frozen blood as well as to establish normal control values the biochemical characteristics of fresh and liquid stored blood have been studied.

In the author's hands the normal values for the intracellular potassium content of freshly drawn red blood cells varied between 8.7 to 11.5 m moles/10^{12} red blood cells with a mean value of 9.91 ± 0.77 n = 20 (Table 1.1). The normal value for the 2,3 diphosphoglycerate (2,3 DPG) content of fresh red-blood cells, collected from healthy individuals, was between 9.8 and 15.2 μ mole/gram haemoglobin with an average value of 13.01 ± 1.79 n = 17 (Table 1.2). However this compound rapidly disappears on liquid storage of the blood in ACD at 4°C, so that after only two days of storage the intracellular 2,3 DPG decreased by about 29% to 9.24 ± 1.28 μ mole/g Hb (n = 12) (Table 3.9). After three days of storage in ACD at +4°C the 2,3 DPG decreased to 57% of its normal value, 7.52 ± 1.46 μ mole/g Hb n = 5 (Table 1.5). By the ninth day of storage it was observed that the intracellular 2,3 DPG had completely disappeared from the red cells (Figure 1.1 and Table 1.5).

As regards the ATP content of red cells the normal values ranged from 2.34 to 4.17 μ mole/g Hb with a mean value of 3.14 ± 0.54 n = 20 (Table 1.3).

Although the concentration of this compound also decreased during storage of ACD blood at 4°C, the rate of its disappearance was slower than that of the 2,3 DPG. The level of ATP gradually declined from

the normal level to 2.84 $\pm$ 0.30 μ mole/g Hb (i.e. 90% of normal) after three days of storage, reaching 2.18 $\pm$ 0.24 (69% of normal) after nine days and becoming 1.16 $\pm$ 0.09 (37% of normal) after fifteen days.

Storage of blood in ACD at 4°C results in a gradual increase in the plasma free haemoglobin and potassium and a decrease in blood pH (Table 1.4). By the end of three weeks storage at 4°C the plasma potassium may be as high as 16-28 m moles/l with a mean value of 21.4 $\pm$ 4.01 (n = 10). Observed plasma haemoglobin, however, has not increased to the same extent as the potassium, and the highest level observed was 0.31 g/l (Table 1.4). We also observed a slightly lower blood pH after three weeks storage than that reported in the literature (Bunker 1966) for fresh ACD blood (Table 1.4). The observed level for the blood pH after three weeks was 6.72 $\pm$ 0.08, whilst the reported level for the fresh ACD blood was 6.9 to 7.0.

TABLE 1.1

Intracellular K^+ concentration of fresh red-blood cells

	Unit No.	K^+ m mol/10^{12} R.B.C.
1.	03674	10.0
2.	03681	9.4
3.	03688	9.5
4.	03687	11.2
5.	03680	10.5
6.	03691	9.4
7.	03685	9.7
8.	03683	9.0
9.	03678	9.5
10.	03675	9.7
11.	03695	11.5
12.	03679	9.0
13.	03701	8.7
14.	03703	10.1
15.	03696	9.4
16.	03684	9.7
17.	03682	10.8
18.	03699	9.8
19.	03697	11.1
20.	03700	10.2
	Mean	9.91
	S.D.	0.77
	n	20

TABLE 1.2

2,3 DPG content of fresh red-blood cells collected from healthy individuals

		2,3 DPG μ mole/g Hb
1.	K.A.A.	14.6
2.	D.S.P.	12.7
3.	S.H.M.	14.2
4.	H.M.R.	15.2
5.	J.Mc.T.	14.5
6.	03688	10.6
7.	03674	15.2
8.	03680	13.4
9.	03687	11.8
10.	03691	9.8
11.	03685	12.5
12.	03681	12.6
13.	47242	10.6
14.	47269	14.0
15.	47115	14.9
16.	47249	14.1
17.	47246	10.6
	Mean	13.017
	S.D.	1.79
	n	17

TABLE 1.3

ATP concentration of fresh red-blood cells collected from healthy individuals

		ATP μ mole/g Hb
1.	03674	2.84
2.	03681	3.28
3.	03688	2.34
4.	03687	2.46
5.	03680	3.01
6.	03691	2.88
7.	03685	3.20
8.	03683	3.04
9.	03678	3.70
10.	03675	2.80
11.	03695	4.06
12.	03679	2.50
13.	03701	3.60
14.	03703	3.70
15.	D.S.P.	2.94
16.	K.A.A.	3.05
17.	S.H.M.	3.76
18.	J.Mc.T.	2.42
19.	H.M.R.	4.17
20.	A.A.S.	3.13
	Mean	3.144
	S.D.	0.539
	n	20

TABLE 1.4

Plasma haemoglobin, plasma potassium and pH of ACD-blood stored at +4°C for three weeks

Pack No.	Age of the blood (days)	free plasma Hb mg/dl	Conc.of K^+ in the plasma (m mol/l)	pH Whole blood
1. 31519	21	22	16	6.7
2. 31734	21	27	21	6.6
3. 31738	21	29	20	6.8
4. 31691	21	31	25	6.8
5. 31749	21	14	18	6.7
6. 32965	21	25	26	6.7
7. 31668	21	16	23	6.8
8. 61718	21	29	28	6.6
9. 32249	21	28	17	6.7
10. 61691	21	28	20	6.8
Mean	21	24.9	21.4	6.72
S.D.	0	5.78	4.01	0.08
n	10	10	10	10

TABLE 1.5

Rate of disappearance of 2,3 DPG and ATP from blood collected in ACD and stored at +4°C

	3 day old		9 day old		15 day old	
	2,3 DPG μ mole/g Hb	ATP μ mole/g Hb	2,3 DPG μ mole/g Hb	ATP μ mole/g Hb	2,3 DPG μ mole/g Hb	ATP μ mole/g Hb
48036	7.0	2.93	0	2.19	0	1.20
47936	8.1	3.02	0	2.32	0	1.27
47944	8.7	2.96	0	2.34	0	1.05
47951	5.2	2.3	0	1.76	0	1.1
47961	8.6	2.97	0	2.30	0	1.2
Mean	7.52	2.84	0	2.18	0	1.16
S.D.	1.46	0.30		0.24		0.09
n	5	5		5		5

Effect of anticoagulant in Freezing

There was no statistically significant difference between the final red cell recoveries of frozen-thawed washed blood collected in ACD anticoagulant and that collected in EDTA, irrespective of the method of deglycerolization used.

There was no statistically significant difference between the supernatant free haemoglobins of frozen-thawed-washed blood that was collected in ACD and that collected in EDTA whether the method of washing was manual or automatic (table 2.5).

There was no statistically significant difference between the supernatant free potassium of frozen-thawed-washed blood that was collected in ACD and that collected in EDTA regardless of the method of washing (Table 2.5).

Also, there was no statistically significant difference between the concentration of intracellular potassium in frozen-thawed-washed erythrocytes that were collected in ACD and those collected in EDTA irrespective of the method of washing (Table 2.5).

Similarly there was no statistically significant difference between the 2,3 DPG content of frozen-thawed-washed erythrocytes that were collected in ACD and those collected in EDTA, whether the blood was washed manually or automatically in the IBM 2991 Cell Processor (Table 2.5).

TABLE 2.1

Comparison of manual and automatic methods

ACD units - manually processed

Unit No.	Age prior to freezing	Age frozen (days)	Recovery(%)	Spnt. Hb (mg/dl)	Spnt. K^+ m (mol/l)	Int. cell K+(m mol/10^{12} RBC)	2,3 DPG (µmol/gHb)	pH
1. 19950	2	23	90.5	290	1.0	N.D.	2.8	6.5
2. 19521	2	23	84	240	0.5	N.D.	1.0	6.5
3. 01968	2	3	96.4	290	1.0	8.8	N.D.	6.8
4. 12277	2	48	96.6	105	1.0	8.4	4.5	N.D.
5. 12353	2	47	96.1	158	1.0	7.4	5.0	N.D.
6. 51560	2	36	84	2,150	8	4.9	9.9	N.D.
7. 19528	2	11	86.4	200	1.0	7.1	N.D.	N.D.
8. 19519	2	11	75.6	860	1.0	7.5	N.D.	N.D.
9. 52521	N.D.	26	96.5	163	6.0	7.9	12.7	N.D.
10. 01965	2	3	96	230	1.0	8.5	N.D.	6.8
11. 01969	2	3	96.3	160	0.5	8.5	N.D.	6.9
12. 01962	2	3	96.3	160	1.0	7.9	N.D.	6.8
13. 01960	2	9	91.2	110	0	8.1	N.D.	6.4
14. 34933	3	16	93.8	210	0.5	8.6	N.D.	6.7
15. 06442	2	14	92.3	250	0.5	7.5	N.D.	N.D.
16. 62101	2	12	92.3	180	1.8	6.7	5.4	6.0
17. 34947	3	16	91.1	230	0.5	7.8	N.D.	6.1
18. 05695	2	32	91.9	370	2.0	6.2	N.D.	6.0
19. 34950	4	13	94.9	86	1.0	6.0	7.0	6.0
20. 06432	3	15	94.5	200	0.5	11.9	N.D.	6.0
21. 06444	2	13	90	300	1.0	5.8	N.D.	6.2
22. 06446	2	70	93.2	80	0.5	6.1	N.D.	6.0
23. 34956	4	71	92.3	120	1.0	6.2	N.D.	6.4
24. 62075	2	13	92.7	100	0.5	7.2	5.8	6.1
25. 05698	2	32	94.1	160	0.5	5.6	N.D.	5.9
26. 62100	2	13	88	1,720	5.0	7.8	7.6	6.0
27. 06428	3	15	89.1	630	2.0	7.0	N.D.	6.0
Mean	2.31	21.89	91.71	361.19	1.49	7.42	6.17	6.31
S.D.	0.62	18.54	4.86	486.84	1.85	1.41	3.37	0.34
n	26	27	27	27	27	25	10	20

* N.D. not determined.

TABLE 2.2

ACD Units - IBM processed

Unit No.	Age prior to freezing	Age frozen (days)	Recovery %	Spnt. Hb mg/dl	Spnt. K+ m mol/l	Intra.K+ m mol/ 10^{12} R.B.C.	2,3DPG μ mole/g Hb	pH
04776	2	7	96.5	87	0.5	7.4	6.1	6.0
05489	5	1	96.1	52	0.5	7.5	N.D.*	6.0
05507	5	1	94.7	200	0.5	6.9	N.D.	6.1
04781	2	7	96.6	67	1.0	6.5	9.2	6.0
06573	2	8	96.5	90	1.0	8.3	N.D.	6.0
06589	2	8	95.8	100	1.0	8.1	N.D.	N.D.
06449	2	7	96.4	400	6.5	8.1	N.D.	6.8
34945	3	69	95	430	5.75	7.4	N.D.	6.6
06588	2	6	95.2	420	0.5	8.6	N.D.	6.8
06594	2	6	95.6	390	0	7.6	N.D.	6.8
06599	2	6	93.6	410	1.0	8.4	N.D.	6.8
06593	2	6	95.6	340	0.5	8.3	N.D.	6.7
06572	2	7	96.6	240	0.5	8.3	N.D.	6.7
06583	2	7	94.3	920	2.0	8.2	N.D.	6.8
06451	2	9	92	2160	3.75	7.1	N.D.	6.8
06584	2	7	95.6	390	0.5	8.8	N.D.	6.8
06591	2	7	95.4	400	1.0	8.9	N.D.	6.8
Mean	2.41	9.94	95.38	417.41	1.56	7.91	7.65	6.53
S.D.	1.0	15.37	1.22	496.85	1.92	0.69	2.19	0.36
n	17	17	17	17	17	17	2	16

* N.D. not determined.

TABLE 2.3

EDTA units manually processed

Unit No.	Age prior to freezing	Age frozen (days)	Recovery(%)	Spnt.Hb (mg/dl)	Spnt.K+ (mmol/l)	Intra.K+ (m mol/ 10^{12} R.B.C.)	2,3DPG (μmole/g Hb)	pH
51344	2	6	92.5	360	4.0	10	N.D.*	N.D.
51387	2	8	94.7	220	2.0	8.1	N.D.	N.D.
52594	3	6	94.6	180	2.0	8.1	N.D.	N.D.
52612	3	6	93.1	350	2.0	6.5	N.D.	N.D.
52258	1	16	95.6	110	2.0	9.3	N.D.	N.D.
52243	1	30	93.4	180	1.0	8.3	3.3	N.D.
52558	3	53	95	430	1.0	5.6	6.4	N.D.
52527	3	47	91.1	600	4.3	5.7	7.3	N.D.
52251	1	47	92.3	430	1.8	4.7	6.0	N.D.
52528	3	53	89.7	960	2.5	5.6	7.8	N.D.
24136	1	12	94.5	470	2.0	8.6	6.2	7
24139	1	12	94.9	290	1.5	6.0	6.2	7
24137	1	12	96.3	200	0.5	11	6.0	7
24142	1	12	92.8	310	2	8.7	5.9	7
20819	2	12	92.1	260	1.0	N.D.	3.3	6.6
20798	2	12	86.4	360	1.0	N.D.	5.5	6.6
24938	1	52	92.7	155	7.5	7.1	5.0	7
26032	N.D.	16	96.8	230	1.0	6.6	4.6	7.1
26021	N.D.	15	95.4	310	0.5	8.9	4.4	7.1
20817	2	62	90.8	300	1.0	9.4	4.7	7.1
20799	2	4	94.7	200	0.5	7.6	15.4	N.D.
20795	2	6	94.8	340	1	7.3	14.6	N.D.
Mean	1.85	22.68	93.37	329.32	1.91	7.66	6.62	6.95
S.D.	0.81	19.51	2.4	182.55	1.6	1.68	3.38	0.19
n	20	22	22	22	22	20	17	10

* N.D. Not determined.

TABLE 2.4

EDTA units IBM processed

Unit No.	Age prior to freezing	Age Frozen (days)	Recovery(%)	Spnt.Hb (mg/dl)	Spnt.K+ (mmol/l)	Intra.K+ (mmol/10^{12} R.B.C.)	2,3DPG μmol/g Hb)	pH
31154	2	8	95.8	150	0.5	7.8	N.D.*	6.2
31151	2	6	96	89	0.5	3.4	11.8	6.1
31163	2	6	96	90	0.2	3.4	11.1	N.D.
31153	2	8	95.8	80	0.5	7.5	N.D.	6.4
24942	1	52	91.0	550	0.5	9.8	5.4	6.8
24947	1	52	93.9	440	0.5	6.9	5.0	6.8
26024	2	9	95	110	0	6.4	10.7	6.7
26025	2	14	90	170	0	5.5	12.6	6.6
26033	2	30	94.5	180	0.5	7	4.3	6.9
27673	2	16	92.8	190	0.5	7.1	4.5	6.8
27661	2	20	93	360	0.5	7.8	5.7	6.7
27653	2	20	95	170	0.5	8.5	5.9	6.8
24946	1	56	95.3	370	0.5	7	9.7	6.8
24939	1	56	94.1	450	0.8	7.7	11.1	7.0
31176	2	13	95.8	60	0	8.7	N.D.	7
31171	2	13	96	120	3	7.8	N.D.	6.7
24141	1	115	94.5	300	0.5	6.3	N.D.	6.8
Mean	1.71	29.06	94.38	228.18	0.54	6.98	8.15	6.69
S.D.	0.47	28.85	1.78	152.34	0.67	1.67	3.25	0.26
n	17	17	17	17	17	17	12	16

* N.D. not determined.

TABLE 2.5

Summary of comparison of the effect of different anticoagulants and the characteristics of the frozen red cells when processed by either the manual or the automatic techniques

		Duration of storage of the blood(days)		Red cell recovery (%)	Supernatant Hb (mg/ dl)	Supernatant K+ (mmol/l)	Intracellular K+ (mmol/10^{12}RBC)	2,3DPG (µmole/g Hb)	pH
		at 4°C before freezing	in the frozen state						
Group I									
ACD units	M	2.07	18.4	91.5	371.7	1.6	7.8	6.0	6.7
manually processed	S.D.	0.27	15.3	6.3	523	2.3	1.01	4.4	0.18
	n.	14	15	15	15	15	13	6	8
Group II	M.	2.41	9.9	95.4	417.4	1.56	7.91	7.65	6.5
ACD units	S.D.	1.0	15.4	1.2	496.8	1.9	0.89	2.19	0.36
IBM processed	n.	17	17	17	17	17	17	2	16
Group III									
EDTA Units	M.	1.9	22.7	93.4	329.3	1.91	7.7	6.6	6.95
manually processed	S.D.	0.8	19.5	2.4	182.5	1.6	1.7	3.4	0.19
	n.	20	22	22	22	22	20	17	10
Group IV									
EDTA units	M.	1.7	29.0	94.4	228.2	0.54	7.0	8.2	6.7
IBM processed	S.D.	0.04	28.8	1.8	152.3	0.67	1.7	3.3	0.26
	n.	17	17	17	17	17	17	12	16

Table 2.5 (cont'd.)

		Duration of storage of the blood (days) at 4°C before freezing	Duration of storage of the blood (days) in the frozen state	Red cell recovery (%)	Supernatant Hb (mg/ dl)	Supernatant K+ (m mol/1)	Intracellular K+ (mmol/10^{12} RBC)	2,3DPG (μ mole/ g Hb	pH
Statistics									
Group I vs. II	t			2.504	0.253	0.056	0.415	0.492	1.052
	p			< 0.05	←		> 0.1 →		
Group III vs. IV	t			1.451	1.841	3.273	1.229	1.216	2.716
	p			> 0.10	< 0.1 > 0.05	< 0.01	> 0.1	> 0.1	< 0.05
Group I vs. III	t			1.290	0.353	0.497	0.235	0.368	3.099
	p			←		> 0.1	→		< 0.02
Group II vs. IV	t			1.909	1.501	2.031	2.388	0.206	1.467
	p			< 0.1 > 0.05	> 0.1	< 0.1 > 0.05	< 0.05	> 0.1	> 0.1

Comparative Study Between Manual and Automatic Techniques of Washing

The Manual method of washing red cells that have been previously frozen with low-glycerol in liquid nitrogen is a standard technique in Britain. However little data have been published concerning the characterization of the process or of the red cells so produced. For this reason and because we have introduced some changes in the original method adopted in Britain, we have studied the manual technique as used in our department as well as the physical and chemical characteristics of the final red cell suspension. Also we have made a comparative study between the standard method of processing and the automatic technique that utilizes the IBM 2991 Automatic Cell Processor.

1. In vitro red cell recovery

The average in vitro red cell recovery obtained by our method is 92.5% with the manual method and 95% with automatic method. In order to obtain this recovery we use approximately equal volumes of packed red cells, about 70% haematocrit, and glycerol solution to give a final concentration of 21% W/V glycerol (Table 3.1 and 3.2).

2. Wash Solutions

As regards the wash solutions used with the manual technique, the total volume ranged from 880-1300 ml, divided into three roughly equal washes, the first is 19% sorbitol while the second and third are 0.9% sodium chloride, (Table 3.1, 3.2, 3.3 and 3.15). We found that in vitro red cell recovery was not significantly correlated with the volume of wash solutions ($r = 0.0099$ $p > 0.1$ $n = 49$).

With the automatic method of washing, the volume of wash solution was about one and half times that used with the manual method (Table 3.3 and table 3.15). This is because of the larger capacity of the processing bag and the necessity of centrifuging it full. For the same reason it was necessary to top up the bag containing the thawed glycerolized blood with approximately 240 ml of wash I (post-thaw predilution). Retrospective studies have shown that this gives a slightly better in vitro red cell recovery than the manual method ($t = 3.2844$ $p < 0.0025$ $n_1 = 34$ $n_2 = 49$ respectively, (Table 3.3 and Table 3.15). Table 3.3 also shows that this improved recovery is due to a reduction in the amount of haemolysis in the subsequent three washes, as there is no significant difference between both methods as regards the freeze-thaw haemolysis ($t = 0.178$ $p > 0.4$ $n_1 = 34$ and $n_2 = 49$ respectively).

Automatic washing not only gave higher red cell recovery but also resulted in better washing. This was evidenced by the residual glycerol and sorbitol in the supernatant of the washed cells. The figures for the glycerol and sorbitol indicated in Tables 3.4 and 3.5 were obtained from estimation made in the supernatant of red-cell suspensions immediately after the end of the washing procedure and before resuspension in any medium. Although the Hct of the product obtained with the automatic method (82.3%) was higher than that with the manual method (69.13%), the glycerol and sorbitol concentrations in the former were significantly lower than those of the latter, 0.19 ± 0.22 and 0.29 ± 0.21 as compared to 0.84 ± 0.26 and 0.76 ± 0.51 ($t = 6.242$ and 2.696 and $P < 0.0005$ and < 0.0125 respectively (Table 3.14, 3.15).

3. Intracellular Potassium Concentration

Intracellular cation content, particularly potassium, is regarded as an indirect indicator of the state of the membrane permeability which is in turn a reflection of the stability of the red-cell membrane. Because of this, and because of the recent speculation about the correlation of the *in vivo* survival of the frozen-thawed and deglycerolized red cells with the intracellular potassium level (Valeri and Runck 1969b) an estimate of the intra-cellular potassium content in our product was made.

There was a significant reduction (20%)(t test = 6.255 $p < 0.0005$ $n_1 = 26$, $n_2 = 20$ respectively) in the intracellular potassium levels of the previously frozen-thawed red cells when deglycerolized by the manual technique, when compared to those of the fresh red blood cells (Table 1.1 and Table 3.6).

There was a significant reduction (21%) (t - test = 9.161 $p < 0.0005$ $n_1 = 29$, $n_2 = 20$ respectively) in the intracellular potassium levels of the previously frozen-thawed red cells when deglycerolized by the automatic method, as compared to those of the fresh red-blood cells (Table 1.1 and Table 3.7). However there was no statistically significant difference between the intracellular potassium content of red cells processed by the manual method (7.94 ± 1.23 m mol/10^{12} R.B.C.) and those washed by the automatic method (7.81 ± 0.8 m mol/10^{12} R.B.C.) (t-test = 0.461 $p > 0.3$ $n_1 = 26$, $n_2 = 29$ respectively) Table 3.14 and 3.15).

4. 2,3 Diphosphoglycerate Content of the Red Cells

In recent years much attention has been given to the 2,3 DPG content of the red cells as it has a direct relation to the oxygen transport function.

A normal level for the 2,3 DPG content of fresh red cells was established for the method used in this study and was 13.01 $\pm$ 1.79 μ mole/g Hb (Table 1.2). We have estimated the 2,3 DPG content of erythrocytes that were previously frozen-thawed and washed with the manual method, and observed that the level of 2,3 DPG was 6.8 $\pm$ 3.4 μ mole/g Hb for red cells stored for 2.65 days at 4°C prior to freezing and 27.4 days in the frozen state, and that after processing the pH was 6.6. Therefore there was a significant reduction (18%) (t - test = 1.941 $p < 0.05$ $n_1 = 26$, $n_2 = 23$) in the levels of 2,3 DPG of red cell that were frozen by the low-glycerol rapid freeze-thaw technique and deglycerolized by the manual method ($n_1 = 26$), when compared to those of the same erythrocytes prior to freezing ($n_2 = 23$) Table 3.8.

Red-blood cells that were stored at 4°C for 1.8 days, glycerolized and frozen according to our protocol, stored for about 25 days, and then thawed and deglycerolized by the automatic technique had a mean 2,3 DPG level of 7.07 $\pm$ 2.23 μ mole/g Hb at a pH of 6.6 (Table 3.9).

There was a significant reduction (23%) (t - test = 3.126 $p < 0.005$ $n_1 = 27$, $n_2 = 12$ respectively) in the levels of 2,3 DPG of the latter cells compared to the same cells prior to freezing.

The method of deglycerolization appeared to have no effect on the 2,3 DPG level as the difference between the <u>levels</u> of the 2,3 DPG of red cells processed by the manual method and those of red cells processed by the automatic method was not statistically significant (t - test = 0.346 $p > 0.4$ $n = 26$) Table 3.14 and Table 3.17. Moreover, there was no statistically significant difference between the <u>percent reduction</u> of 2,3 DPG following glycerolization, freezing, storage, thawing when processed by either the manual or the automatic method

(t - test = 0.3663 $p > 0.35$ $n_1 = 22$ and $n_2 = 11$ respectively), Table 3.17. Finally, there was no significant correlation between the period of storage in the frozen state and the level of 2,3 DPG recovered by either the manual or the automatic method ($r = 0.380$ and 0.0696 respectively and $p > 0.05$ and > 0.1).

5. Adenosine triphosphate content of the frozen red cells

The importance of estimation of the ATP content in stored blood arises from its role in maintaining the erythrocyte discoid shape, elasticity and viability (Mollison 1972). Moreover a depletion of the red cell ATP results in loss of lipid content of the cell membrane. A normal level of the red cell ATP, as measured in fresh blood by the method used in this study, was 3.144 ± 0.54 μ mole/ g Hb (Table 1.3). However, on storage of the blood at $4^{\circ}C$ there was a slow and gradual decrease in its ATP content over the two weeks of study (Table 1.5)

The ATP content of red cells that were collected in CPD and stored at $4^{\circ}C$ for 4.4 days, frozen and stored at $-180^{\circ}C$ for 48.6 days then thawed and deglycerolized with the manual method was 1.5 ± 0.38 μmol/g Hb. There was a significant decrease (t - test = 6.236 $p < 0.0005$ $n = 10$) in the levels of ATP of erythrocytes that were frozen-thawed and deglycerolized by the manual method, when compared with those of the same cells before freezing (table 3.10).

The ATP content of red cells that were stored for 3 days at $4^{\circ}C$ in ACD, frozen and stored at $-180^{\circ}C$ for 23.6 days, then thawed and deglycerolized with the automatic method was 1.9 ± 0.35 μ mole/g Hb. This level is lower than that estimated for the same cells prior to freezing; the difference was statistically significant (t - test = 3.396 $p < 0.005$ $n = 10$) Table 3.11.

6. Mean Red Cell Corpuscular Volume

In this work the red cell mean corpuscular volume was considered as an approximate indicator of the state of fragility of the cells. Throughout the whole work the MCV was determined by a Coulter Counter Model S. The normal range of MCV as determined by the Coulter was from 80 to 94 (fl) for males and from 81-99 (fl) for females.

The mean MCV for the red cells prior to freezing was 90.1 fl $\pm$ 4.26 n = 88 (Table 4.25 and 4.26). The mean MCV for red cells previously frozen, thawed and then deglycerolized with the manual method was 93.22 fl $\pm$ 4.52 n = 37 (Table 3.12). The mean MCV of red cells frozen, thawed and then washed by the automatic method in the IBM 2991 was 91.41 $\pm$ 6.11 fl n = 34 (Table 3.13). The difference between the values of the MCV of cells processed manually and those of cells processed by the IBM 2991 was not statistically significant (t - test = 1.415 $p > 0.05$ $n_1 = 37$, $n_2 = 34$) Table 3.14. Preliminary results showed that when blood is badly processed the final resuspended cells may have an MCV in excess of 100 fl.

7. Aluminium Content of Blood Frozen in Aluminium Cans

Recent interest in the toxic effects of hyperaluminaemia (Berlyne et al 1970 and Waldron-Edward et al 1971), has concerned those who freeze, store and thaw blood in aluminium cans. However, no published data is available on this subject. For this reason we have investigated the aluminium content in our final product.

The mean aluminium level in the processed blood was found to be $\leqslant 11.1/\mu$ mol/l (n = 33) Table 3.19), as determined by Atomic Absorption Spectroscopy. The normal level in blood (published for the same method) varies from 7 to $11/\mu$ mol/l (Berlyne et al 1970 and Waldron-Edward et al 1971).

TABLE 3.1

Summary of solution volumes used and per cent haemolysis obtained at each processing step of manual washing

		No.of experiments	Mean	Range
1.	Input red cell volume (ml)	63	205	164-270
	Input packed cell volume (%)		0.69	0.4-0.85
2.	Glycerol volume added (ml)	63	191±7.5	171-202
	Final glycerol concentration(% W/V)		20.8±1.6	18.0-23.8
3.	Volume of thawed blood (ml)	63	406	362-468
	Volume of predilution added (ml)		0	0
4.	Post-thaw supernatant volume (ml)	49	217±45	80-305
	Freeze-thaw haemolysis (%)	49	2.5±2.7	1.0-14.8
5.	Sorbitol wash supernatant volume (ml)	49	350±42	260-440
	Haemolysis after sorbitol washing (%)	49	1.13±0.64	0.5-3.8
6.	Saline wash (1) supernatant volume(ml)	49	351±45	265-440
	Haemolysis after first saline washing(%)	40	1.97±1.34	0.6-6.1
7.	Saline wash (2) supernatant volume(ml)	49	337±41	260-430
	Haemolysis after second saline washing (%)	49	1.96±1.61	0.6-7.7
8.	Cellular Hb recovered (g)	49	53.4 ± 6.9	36.1-67.8

N.B. items No. 1,2 and 3 are the same as those in table 3.2.

TABLE 3.2

Summary of solution volume used and per cent haemolysis obtained at each processing step of automatic washing

		No.of experiments	Mean	Range
1.	Input volume of packed cells (ml)	63	205	164-270
	Input packed cell volume haematocrit	63	0.69	0.40-0.85
2.	Glycerol volume added (ml)	63	191±7.5	171-202
	Final glycerol concentration(%w/v)		20.8±1.6	18.0-23.8
3.	Volume of thawed blood (ml)	63	406	362-468
	Volume of predilution added (ml)		240	182-288
4.	Supernatant volume from predilution spin (ml)	34	418±39	350-470
	Freeze thaw haemolysis (%)		2.41±0.61	1.4-3.8
5.	Sorbitol wash supernatant volume (ml)		463±49	230-520
	Haemolysis after sorbitol washing (%)	34	0.44±0.27	0.3-1.9
6.	Saline wash (1) supernatant volume (ml)		442±53	295-510
	Haemolysis after first saline washing(%)	34	1.03±0.66	0.3-3.6
7.	Saline wash (2) supernatant volume (ml)		448±34	400-510
	Haemolysis after second saline washing(%)	34	1.27±0.71	0.6-4.1
8.	Cellular Hb recovered (g)	34	50.8±6.8	31-63

N.B. Items No. 1,2 and 3 are the same as those in Table (3.1).

TABLE 3.3

Summary of comparison of volumes of wash solutions, haemolysis and recovery of red cells processed by the automatic and manual procedures

		Total Hb recovered	Post-thaw volume (ml)	Wash I volume ml	Wash II volume (ml)	Wash III volume (ml)	Total volume of wash solution(ml)	Freeze-thaw haemolysis (%)	haemolysis after Wash I (%)	haemolysis after Wash II (%)	haemolysis after Wash III (%)	Overall recovery (%)
IBM Processed	M	50.8	191	463	442	449	1575	2.4	0.44	1.0	1.27	94.8
	SD	6.82		49	54	35	110	0.6	0.27	0.6	0.7	1.5
	n	34		34	34	34	34	34	34	34	34	34
Manually Processed	M	53.4	217	350	351	337	1038	2.5	1.13	1.97	1.96	92.5
	SD	6.9	45	42	45	41	103	2.7	0.06	1.3	1.6	4
	n	49	49	49	49	49	49	49	49	49	49	49
	t	1.39168		11.192	8.358	12.951	22.762	0.179	5.821	3.743	2.583	3.284
	p	$>.05$		<0.0005	<0.0005	<0.0005	<0.0005	>0.4	<0.0005	<0.0005	<0.01	<0.0025

TABLE 3.4

Glycerol and sorbitol concentration in the supernatant of previously frozen-thawed-manually washed red blood cells

	Unit No.	Glycerol Conc.in the supernatant g/100 ml	Sorbitol conc.in the supernatant g/100 ml	Hct
1.	62100	0.73	0.68	0.717
2.	06428	0.67	0.64	0.690
3.	06444	1.26	1.18	0.686
4.	62075	0.48	0.52	0.646
5.	06442	0.92	0.22	0.841
6.	06432	0.62	0.32	0.603
7.	34947	0.68	0.22	0.724
8.	34933	0.84	0.16	0.759
9.	34950	0.58	0.83	0.690
10.	05695	0.94	1.31	0.643
11.	05698	1.07	1.44	0.669
12.	62101	1.26	1.59	0.628
	Mean	0.84	0.76	0.6913
	S.D.	0.26	0.51	0.643
	n	12	12	12

N.B. The glycerol and sorbitol were measured in the supernatant or red-cell suspensions after the second saline wash, and before resuspension in any other medium, with a haematocrit as indicated in the Table.

TABLE 3.5

Glycerol and sorbitol concentration in the supernatant of previously frozen-thawed automatically washed red-blood cells

	Unit No.	Glycerol conc.in the supernatant (g/100 ml)	Sorbitol conc.in the supernatant (g/100 ml)	Hct
1.	06572	0.095	0.16	0.833
2.	06584	0.128	0.182	0.877
3.	06583	0.146	0.350	0.892
4.	06591	0.13	0.19	0.824
5.	06588	0.127	0.20	0.861
6.	06593	0.120	0.17	0.823
7.	06594	0.160	0.20	0.849
8.	06599	0.03	0.12	0.798
9.	06451	0.816	0.60	0.906
10.	06589	0.117	0.756	0.568
	Mean	0.19	0.29	0.823
	S.D.	0.22	0.21	0.0957
	n	10	10	10

N.B. Glycerol and sorbitol were estimated in the supernatant of red-cell suspensions immediately after the second saline wash, and before resuspension in any other medium, with a haematocrit as indicated in the Table.

TABLE 3.6

Intracellular potassium concentration of red-blood cells processed by the manual method

	Unit No.	Intracellular K^+ m mol/10^{12} R.B.C.
1.	52258	9.3
2.	52612	6.5
3.	52594	8.1
4.	51387	8.1
5.	51344	10.0
6.	26032	6.6
7.	24938	7.1
8.	12277	8.4
9.	12353	7.4
10.	52243	8.3
11.	52521	7.9
12.	20795	7.3
13.	19519	7.5
14.	19528	7.1
15.	20799	7.6
16.	26021	8.9
17.	20817	9.4
18.	34933	8.6
19.	06442	7.5
20.	05695	6.2
21.	34947	7.8
22.	62101	6.7
23.	06432	11.9
24.	62075	7.2
25.	62100	8.0
26.	06428	7.0
	Mean	7.94
	S.D.	1.23
	n	26

TABLE 3.7

Intracellular potassium concentration of red-blood cells processed by the automatic technique (IBM)

	Unit No.	Intracellular K^+ m mol/10^{12} R.B.C.
1.	24942	9.8
2.	24947	6.9
3.	24946	7.0
4.	24939	7.7
5.	27673	7.1
6.	26033	7.0
7.	27661	7.8
8.	26753	8.5
9.	31176	8.7
10.	06591	8.9
11.	06584	8.8
12.	06451	7.1
13.	06583	8.2
14.	06572	8.3
15.	06593	8.3
16.	06599	8.4
17.	06594	7.6
18.	06588	8.6
19.	24141	6.3
20.	34945	7.4
21.	06449	8.1
22.	06589	8.1
23.	06573	8.3
24.	04781	6.5
25.	05507	6.9
26.	05489	7.5
27.	04776	7.4
28.	31154	7.8
29.	31153	7.5
	Mean	7.81
	S.D.	0.801
	n	29

TABLE 3.8

2,3 DPG content of previously frozen red-blood cells processed by the manual method

	Unit No.	Duration of storage (days)		2,3DPG(μmole/gHb)		pH
		at 4°C before	in the frozen state	before freezing	after processing	
1.	26032	N.D.	16	N.D.	4.6	7.0
2.	52243	1	30	11.1	3.3	N.D.
3.	12353	2	50	9.4	5.0	"
4.	12277	2	47	9.3	4.5	"
5.	24938	1	28	11.5	4.0	7.0
6.	19521	3	24	7.3	1.0	6.5
7.	19550	3	24	7.7	3.6	6.5
8.	20798	4	13	6.0	5.5	6.6
9.	20819	4	13	5.1	3.3	6.6
10.	51560	3	36	7.5	9.9	N.D.
11.	52558	3	22	8.0	6.3	"
12.	52527	4	16	9.0	8.1	"
13.	52251	2	16	7.5	6.1	"
14.	20817	3	63	7.9	4.8	7.1
15.	26021	N.D.	15	N.D.	4.5	7.1
16.	20799	3	63	8.1	15.3	N.D.
17.	52528	3	53	7.8	7.9	"
18.	20795	3	66	7.0	14.6	"
19.	52521	N.D.	28	N.D.	12.7	6.0
20.	62101	3	12	9.1	8.4	6.0
21.	62075	3	13	7.1	5.7	6.0
22.	62100	3	13	8.1	7.9	6.0
23.	24136	2	13	8.2	7.2	6.9
24.	24137	2	13	9.5	7.5	6.9
25.	24142	2	13	9.3	7.5	6.9
26.	24139	2	13	8.9	7.5	N.D.
	Mean	2.65	27.4	8.28	6.8	6.6
	S.D.	0.83	18.05	1.44	3.4	0.45
	n	23	26	23	26	15

TABLE 3.9

2,3 DPG content of previously frozen red-blood cells processed by the automatic method

	Unit No.	Duration of storage (days)		2,3DPG(μmole/g/Hb)		pH
		before freezing at 4°C	in the frozen state	Before freezing	after processing	
1.	24942	1	53	N.D.	6.0	6.8
2.	24947	1	53	"	5.0	6.8
3.	24946	1	57	"	9.5	6.8
4.	24939	1	57	"	11.0	6.9
5.	27673	2	17	"	4.5	6.7
6.	26033	N.D.	17	"	4.3	6.9
7.	26753	2	21	"	5.8	6.8
8.	26661	2	21	"	5.7	6.7
9.	26025	2	14	"	9.4	6.6
10.	26024	2	14	"	8.7	6.6
11.	04781	2	7	"	9.2	6.0
12.	04776	2	7	"	6.0	6.0
13.	31151	2	7	"	11.7	6.1
14.	31163	2	7	"	11.1	6.1
15.	47242	2	27	7.9	4	6.3
16.	47269	2	27	10.4	7.3	6.5
17.	47115	3	27	10.3	6.8	6.4
18.	47249	2	27	10.5	7.1	6.4
19.	47246	2	27	7.9	5.5	6.4
20.	53507	2	20	7.9	7.0	6.8
21.	53479	2	20	11.3	8.2	6.8
22.	53475	2	20	9.9	7.5	6.6
23.	53492	2	20	N.D.	4.6	6.7
24.	53468	2	26	8.8	N.D.	6.8
25.	53467	2	26	7.3	4.1	6.8
26.	53959	2	26	9.3	7.2	6.7
27.	53953	2	26	9.4	6.6	6.6
	Mean	1.77	24.8	9.24	7.069	6.6
	S.D.	0.65	14.5	1.28	2.232	0.28
	n	26	27	12	26	27

TABLE 3.10

ATP content of the red cells prior to freezing and after processing by the manual technique

	Unit No.	Duration of storage (days)		ATP conc. μ mole/g Hb	
		at 4°C before freezing	in the frozen state	Prior to freezing	after processing
1.	51868	5	9	2.6	1.5
2.	51992	4	9	2.85	2.0
3.	51993	4	9	1.87	1.1
4.	51998	4	9	2.62	2.0
5.	66696	4	75	2.42	1.9
6.	66714	4	75	2.44	1.7
7.	66720	4	75	2.58	1.4
8.	14327	5	75	2.37	1.4
9.	14329	5	75	1.24	1.1
10.	14330	5	75	1.15	1.0
	Mean	4.4	48.6	2.21	1.5
	S.D.	0.52	34.1	0.59	0.38
	n	10	10	10	10

TABLE 3.11

ATP content of red cells prior to freezing and after processing by the automatic technique

	Unit No.	Duration of storage (days)		ATP (µ mole/g Hb)		pH
		at 4°C before freezing	in the frozen state	prior to freezing	after processing	
1.	53507	3	20	2.6	2.0	6.8
2.	53479	3	20	2.5	2.1	6.8
3.	53475	3	20	2.6	1.8	6.6
4.	53492	3	20	2.0	1.7	6.7
5.	52468	3	26	2.6	1.7	6.8
6.	53467	3	26	3.4	1.1	6.8
7.	53959	3	26	2.6	2.1	6.7
8.	53953	3	26	2.8	2.3	6.6
9.	53951	3	26	2.7	2.2	6.8
10.	10512	3	26	5.0	2.0	6.5
	Mean	3	23.6	2.88	1.9	6.71
	S.D.	0	3.1	0.82	0.35	0.11
	n	10	10	10	10	10

TABLE 3.12

Mean corpuscular volume and white cell content of previously frozen red-blood cell suspension processed by the manual method

	Unit No.	MCV(fl)	W.B.C.x (10^6/g Hb)		Unit No.	MCV(fl)	W.B.C.x (10^6/g Hb)
1.	19550	100	2.04	30.	20819	92	1.92
2.	19521	107	0.83	31.	20798	95	1.18
3.	12277	94	1.2	32.	24938	94	1.10
4.	12353	89	1.32	33.	26032	89	2.57
5.	51560	103	3.3	34.	26021	90	1.11
6.	19528	94	5.55	35.	20817	83	0.44
7.	19519	97	4.73	36.	20799	93	3.32
8.	52521	88	3.17	37.	20795	91	2.6
9.	62101	92	2.87		Mean	93.22	2.85
10.	34947	96	1.27		S.D.	4.52	2.15
11.	05695	87	2.38		n.	37	37
12.	06432	93	1.57				
13.	06444	90	0.87				
14.	62075	90	1.43				
15.	05698	95	4.87				
16.	62100	97	1.33				
17.	06428	93	3.10				
18.	34933	94	1.25				
19.	06442	96	0.76				
20.	51344	95	6.85				
21.	51387	90	3.64				
22.	52954	88	5.11				
23.	52612	94	5.74				
24.	52258	95	1.97				
25.	52243	92	2.20				
26.	52558	87	2.74				
27.	52527	97	2.72				
28.	52251	96	11.11				
29.	52528	93	5.48				

N.B. (1) mean MCV for red cells prefreeze is 90.1 (fl)
(2) mean Hb content of one unit 53.4 g.

TABLE 3.13

Mean corpuscular volume and white cell content of previously frozen red-blood cell suspension processed by the automatic method

Unit No.	MCV (fl)	W.B.C. (10^6/g Hb)	Unit No.	MCV (fl)	W.B.C. (10^6/g Hb)
04776	86	4.42	26033	80	3.43
05489	91	1.65	26025	85	1.57
05507	92	3.5	26024	83	2.87
04781	83	4.86	24947	91	2.4
06573	96	4.06	24942	96	4.43
06589	92	4.3	Mean	91.41	2.52
06449	94	1.12	S.D.	6.11	1.26
34945	95	3.84	n.	34	34
06588	100	2.25			
06594	93	2.23			
06599	96	1.59			
06593	92	1.53			
06572	92	0.38			
06583	103	1.45			
06451	91	0.36			
06584	101	1.10			
06591	101	0.77			
31153	89	2.52			
31163	92	1.98			
31151	90	3.66			
31154	97	2.02			
24141	88	1.15			
31171	92	2.55			
31176	85	4.57			
24939	94	1.55			
24946	94	2.83			
27653	93	3.0			
27661	87	3.1			
27673	74	2.52			

N.B. (1) mean MCV for red cells prefreezing is 90.1 (fl)
(2) mean Hb content of one unit is 50.8 g.

TABLE 3.14

Summary of biochemical and haematological parameter in manual and IBM processed blood

		Residual glycerol (g/100ml)	Residual Sorbitol (g/100ml)	Intracellular K^+ (m mol/10^{12}RBC)	M.C.V. (fl)	Residual leucocyte content x(10^6/gHb)	2,3 DPG (μ mole/g Hb)	ATP (μ mole/g Hb)	24-hour post-transfusion survival(%)
IBM Processed	M	0.186	0.29	7.81	91	2.5	7.1	1.90	95.22
	S.D.	0.22	0.21	0.801	6.0	1.2	2.23	0.35	4.26
	n.	10	10	29	34	34	26	10	5
Manually-Processed	M	0.84	0.76	7.94	93	2.85	6.8	1.5	93.38
	S.D.	0.26	0.51	1.23	4.5	2.15	3.4	0.38	6.15
	n	12	12	26	37	37	26	10	5
Statistics	t	6.242	2.696	0.461	1.415	0.799	0.346	2.058	0.523
	p	<0.0005	<0.0125	>0.3	>0.05	>0.2	>0.35	<0.05	>0.3

TABLE 3.15

Overall characteristics of both methods, manual versus automatic

	Manually processed			IBM processed		
	Mean	S.D.	n.	Mean	S.D.	n.
Supernatant volume from predilution spin (ml)	217 ±	45	49	418 ±	39	34
Predilution volume (ml)	N.D.	N.D.	N.D.	227		
Post-thaw haemolysis (%)	2.5 ±	2.7	49	2.4 ±	0.6	34
Sorbitol wash supernatant volume(ml)	350 ±	42		463 ±	49	
Sorbitol wash haemolysis (%)	1.13 ±	0.6		0.44 ±	0.27	
Saline wash(1)supernatant volume(ml)	351 ±	45		442 ±	54	
Saline wash(1)haemolysis (%)	1.97 ±	1.3		1.0 ±	0.6	
Saline wash(2)supernatant volume(ml)	337 ±	41		449 ±	35	
Saline wash (2) haemolysis (%)	1.96 ±	1.6		1.27 ±	0.7	
Total volume of wash solutions(ml)	1038 ±	103	49	1575 ±	110	
Total haemolysis (%)	7.5			5.2		
Overall recovery (%)	92.5 ±	4		95 ±	1.5	
Residual glycerol (g%)	0.84 ±	0.26	12	0.19 ±	0.22	10
Residual sorbitol (g%)	0.76 ±	0.51	12	0.29 ±	0.21	10
Intracellular K^+ (m mole/10^{12} R.B.C.)	7.9 ±	1.2	27	7.8 ±	0.8	29
Mean corpuscular volume (MCV) fl	93 ±	4.5	37	91 ±	6.0	34
Residual leucocyte content(10^6/g Hb)	2.85 ±	2.15	37	2.5 ±	1.2	34
Cellular 2,3 DPG content(μ mole/gHb)	6.8 ±	3.4	26	7.1 ±	2.2	26
Cellular ATP content (μ mole/g Hb)	2.01 ±	0.43	10	1.90 ±	0.35	10
24-hour post-transfusion survival(%)	93.38 ±	6.15	5	95.22 ±	4.26	5

Time required to process one unit (minutes)	90	20
Time required to process four units (minutes	120	120
Capital cost	N.D.	£9,000
Disposable harness cost	£1.00p	£3.80p

TABLE 3.16

Effect of glycerolization, freezing, thawing and washing on the organic phosphate content, on the leucocyte count and red cell mean corpuscular volume

Unit No.	Duration of storage at 4°C before freezing	Duration of storage in the frozen state	pH Post-washing	2,3 DPG µ moles/g Hb		ATP µ moles/gHb		W.B.C.x 10^6 per g Hb		M.C.V. fl	
				before	after	before	after	before	after	before	after
10512	3	54	6.5	6.5	1.3	5.0	2	93.7	2.1	79	95
45026	7	48	6.6	2.8	0	2.4	1.5	29.5	1.7	88	92
44936	8	50	6.5	0	0	1.5	1.33	112.5	0.83	100	94
63628	14	50	6.7	0	0	3.5	1.9	22.4	1.78	88	97
44395	15	48	6.6	0	0	1.87	1.3	23.6	4.9	90	81
09719	17	54	6.9	0	0	3.3	2.7	38.2	2.1	88	92
63585	18	48	6.6	0	0	1.6	1.4	22.5	3.8	92	102
43914	19	48	6.3	0	0	4.4	1.4	30	0.9	94	90
53507	3	20	6.8	7.9	7.0	2.6	2.0	41.2	1.9	106	107
53479	3	20	6.8	11.3	8.2	2.5	2.1	36.3	3.1	91	93
53475	3	20	6.6	9.9	7.5	2.6	1.8	64.5	8.0	101	101
53492	3	20	6.7	N.D.	4.6	2.0	1.7	30.4	7.3	91	94
53468	3	26	6.8	8.8	N.D.	2.6	1.7	34.8	N.D.	105	N.D.
53467	3	26	6.8	7.3	4.13	3.4	1.1	40.7	3.7	96	96
53959	3	26	6.7	9.3	7.2	2.6	2.1	56.8	2.9	91	91
53953	3	26	6.6	9.4	6.6	2.8	2.3	75.7	2.5	91	92
53951	3	26	6.8	5.3	N.D.	2.7	2.2	100	N.D.	89	N.D.
Mean	7.53	35.88	6.66	7.85	5.17	2.79	1.8	50.16	3.17	92.94	94.47
S.D.	6.3	14.01	0.15	2.49	2.9	0.92	0.42	28.91	2.12	6.84	5.97
n.	17	17	17	10	9	17	17	17	15	17	15

All units were processed by the IBM 2991 and resuspended in isotonic saline.

TABLE 3.17

Summary of the effect of glycerolization, freezing, thawing and washing on the 2,3 DPG content of the red cells

		Period of storage at 4°C before freezing (days)	Period of storage in the frozen state (days)	2,3 DPG(μmoles/gHb)		(%) Reduction	pH
				Before freezing	after washing		
IBM processed	M	1.77	24.8	9.24	7.06	30.5	6.58
	S.D.	0.65	14.5	1.28	2.23	10.15	0.28
	n.	26	27	12	26	11	27
Manually processed	M	2.65	27.4	8.24	6.8	24.9	6.6
	S.D.	0.83	18.05	1.44	3.4	24.1	0.45
	n.	23	26	23	26	22	15
	t			0.34591	0.3459	0.3663	
	p			>0.3	>0.35	>0.35	

TABLE 3.18

Effect of glycerolization, freezing, storage, thawing and processing on the ATP content of the red cells

		Storage period at 4°C before freezing (days)	Storage period in the frozen state (days)	ATP/μ moles/g Hb		% Reduction	pH
				Before freezing	after washing		
Manually processed	M	4.4	48.6	2.21	1.5	29.9	7.0
	S.D.	0.52	34.08	0.59	0.38	12.5	0.1
	n.	10	10	10	10	10	10
IBM processed	M	3	23.6	2.88	1.9	30.4	6.71
	S.D.	0	3.10	0.82	0.35	18.9	0.11
	n.	10	10	10	10	10	10
	t			1.648	2.058	0.062	
	p			$< 0.1 > 0.05$	< 0.05	> 0.4	

TABLE 3.19

Aluminium content of blood frozen and thawed in aluminium cans
(Atomic Absorption Spectroscopy)

	Unit No.	Aluminium Concentration /u mol/l		Unit No.	Aluminium Concentration /u mol/l
1.	52258	218			
2.	MH12	33.3	25.	55828	< 7.4
3.	MH13	103.7	26.	53950	< 7.4
4.	MH18	44.4	27.	53951	< 7.4
5.	51344	26.6	28.	13101	< 7.4
6.	51387	26.6	29.	12754	< 18.5
7.	52594	11.8	30.	34918	< 7.4
8.	52612	11.8	31.	13142	< 7.4
9.	52243	< 7.4	32.	53926	< 7.4
10.	52521	< 7.4	33.	53930	< 7.4
11.	51560	< 7.4			
12.	12353	8.8			
13.	12277	28.1			
*14.	53468	< 7.4			
15.	53507	14.8			
16.	53475	< 7.4			
17.	53479	< 14.8			
18.	53467	< 11.1			
19.	53959	11.1			
20.	53953	< 7.4			
21.	14285	< 7.4			
22.	53492	< 7.4			
23.	51920	< 37			
24.	12750	< 7.4			

* The method of analysis was improved at the Laboratory of the Government Chemist to exclude artifacts which are apparent in the 13 early samples.

POST-THAW STABILITY OF PREVIOUSLY FROZEN-THAWED-WASHED ERYTHROCYTES

It was observed that haemolysis of previously frozen-thawed-washed erythrocytes increases continuously during post-thaw storage at +4°C (Fig. 4.1 and Tables 4.1 and 4.8). The maximum rate of haemolysis occurs during the first 24 hours after which it continues to rise at about half the initial rate. The level of free Hb in the supernatant of cells resuspended in isotonic saline was found to be 370,650, and 1010 mg/dl at 4 hours, 24 hours, and 9 days after processing, respectively (Table 4.1). Due to technical limitations, zero time values were actually performed 4 hours after thawing and washing.

Because of this poor in vitro stability of the processed cells and because of the high risk of bacterial contamination, the shelf-life of these frozen-thawed-washed red cells was restricted to 12-24 hours post-processing. Although this product, when used in this way, proved to be clinically acceptable it presented difficulties for efficient blood bank management, because frozen cells cannot readily be made available on an emergency basis without significant wastage. Moreover, long-distance transport was severely restricted. For these reasons a series of studies were designed to find a suitable medium in which the shelf life of frozen-thawed-washed red cells destined for transfusion can be significantly prolonged.

Different resuspension media were found to have different effects on the rate of haemolysis (Table 4.12 and Table 4.13). We observed that 5% W/V albumin solution in isotonic saline was superior to 0.9% W/V saline, Ringer's lactate solution and "Tis-U-Sol" in lowering the

level of free Hb in the supernatant (Fig. 4.3 and Table 4.12). The addition of 70 ml ACD to 200 ml of previously frozen-processed erythrocytes lowered the pH of the blood from 6.9 $\pm$ 0.1 to 6.1 $\pm$ 0.1 and at the same time reduced significantly the amount of haemolysis, even after only four hours of storage. Thus the free Hb was reduced to 98 mg/dl at 4 hours post-processing and was only 390 mg/dl at nine days (Fig. 4.1 and Table 4.5). There was no significant difference (t-test = 0.0563 $p > 0.48$ $n_1 = 8$, $n_2 = 10$ respectively) between the concentrations of free potassium in the supernatant of cells suspended in saline and those of cells resuspended in saline + ACD at zero day of processing (Fig. 4.2 and Table 4.13). However, from the first day of post-processing storage through to the ninth day there was a significant difference in the levels of free potassium in the supernatants of cells resuspended in saline + ACD when compared to those supernatants of cells resuspended in saline alone (Table 4.13).

When ACD and albumin (5% w/v in isotonic saline) were combined, the results showed a further improvement with a free Hb of 60 mg/dl and free potassium of 0.5 m mol/l at 4 hours post-processing and 280 mg/dl and 10.2 m mol/l after nine days of storage (Fig. 4.1, Fig. 4.2, Tables 4.6, 4.8, 4.12 and 4.13). Obviously the presence of glucose has little or no effect on this improved stability (Fig. 4.4a and Fig. 4.4b).

The effect of pH was then investigated by observing the post-thaw stability of different groups of previously frozen-processed erythrocytes, each group had a different blood pH, (pH 6.1, 6.5, and 6.8), but all groups were finally resuspended in isotonic saline alone. The required pH was achieved by adding different amounts of ACD or CPD to

wash I, which was subsequently washed out during processing. The results of these experiments are shown in (Fig. 4.5 and Tables 4.5, 4.9, 4.10, 4.11 and 4.14). It was observed that lowering the pH of the blood was accompanied by a reduction in the rate of haemolysis as evidenced by the decrease in the supernatant free Hb.

Although reduction of the pH to 6.1 had a beneficial effect, it is still not as good as that observed in the presence of citrate ions. Thus at pH 6.1, blood which contained ACD in the resuspension medium had a lower rate of haemolysis on storage than that of blood at the same pH without ACD (Fig. 4.5).

The effect of citrate at different pH values was studied separately by adding 70 ml of CPD to previously frozen/thawed/washed erythrocytes and compared the results to those obtained previously with ACD. It was observed that despite the fact that CPD has the same concentration of citrate per unit (36 m mol/l final concentration) as ACD, the latter still gave better results as judged by the rate of haemolysis (Fig. 4.6 and Tables 4.15 and 4.16). It is to be noted that all the above results were obtained with blood processed by the IBM 2991 Automatic Cell Processor, however, almost the same effect was observed with units processed by the manual technique (Tables 4.17 - 4.24).

Evaluation of Previously Frozen-Thawed-Washed Erythrocytes Resuspended In Saline - ACD Medium:

It was evident that the best post-thaw stability results were obtained on resuspension of the washed cells in either albumin-ACD or saline-ACD media, although the latter was preferred for reasons of availablility, sterilization and lower cost. We have investigated the

haematological and biochemical characteristics of red-blood cells that were resuspended in saline-ACD medium after being frozen-thawed and washed. We have also investigated the incidence of bacterial contamination in this product over a period of ten days of post-thaw storage at $+4^{\circ}C$. A total of one hundred units were used in this study and the results of the individual units are shown in Table 4.25, while Table 4.26 shows a summary comparison between the characteristics before and after processing.

Although the mean red cell volume of the processed resuspended cells was within the normal range there was a significant difference (t-test = 4.4307 $p < 0.0005$, $n_1 = 94$, $n_2 = 88$, respectively) between these levels when compared with those of the same cells before freezing.

There was a significant reduction (t-test = 20.324 $p < 0.0005$ $n_1 = 95$, $n_2 = 88$ respectively) in the white cell count in the processed resuspended blood when compared to those of the same blood before freezing. It might be observed that the leucocyte content of this blood was higher than that obtained previously (Table 3.13 and 3.16). This difference may be explained on basis of the method of sampling - while the previous units were sacrificed and samples were obtained from the bag itself, samples in the present series were taken from the harness line of the processing set, which contains most of the buffy coat.

There was a 25% reduction in the levels of the 2,3 DPG content of the red cells due to the processes of freezing, thawing and washing. Thus the intraerythrocytic levels of 2,3 DPG of three day old blood was $7.33 \pm 3.3\mu$ mole/g Hb before freezing and was reduced to 5.53 ± 3.16 after freezing, storage for 42 days, thawing, washing and

resuspension in saline-ACD medium. This difference was statistically significant (t-test = 3.7542 $p < 0.0005$ $n_1 = 87$, and $n_2 = 96$ respectively). However, after storage of this blood at $+4^{\circ}C$, there was a further decrease in the 2,3 DPG content and the levels reached 2.4 μ mole/g Hb after ten days (mean of 15 experiments).

There was also a reduction in the ATP content of the red cells, after processing and resuspension in saline-ACD medium, by about 11% from that of the same cells before freezing. Again this reduction was statistically significant (t-test = 3.0712 $p < 0.0025$ $n_1 = 93$ $n_2 = 85$ respectively). On storage of this blood at $+4^{\circ}C$ there was a further decrease in the intraerythrocytic ATP and after ten days the mean ATP content was 0.99 μ mole/g Hb $n = 15$.

Fig. 4.1

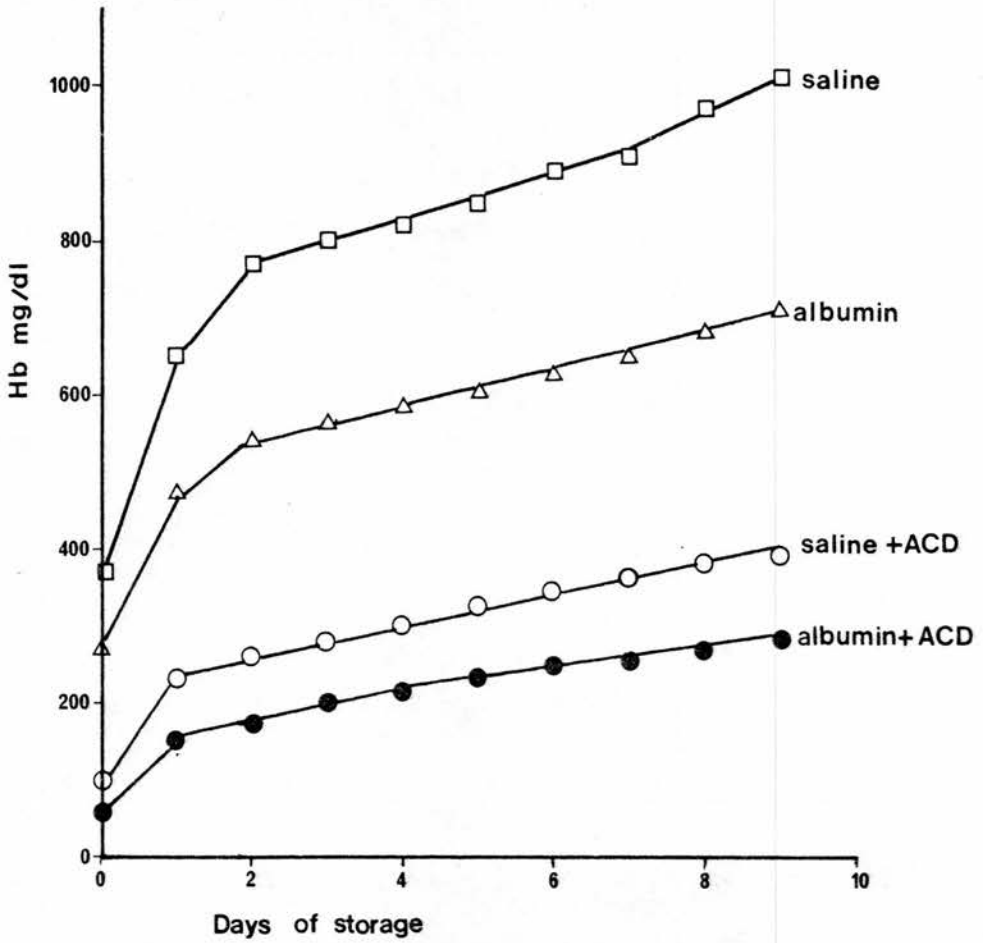

Hb mg/dl
1000
800
600
400
200
0
0
2
4
6
8
10
Days of storage
saline
albumin
saline +ACD
albumin+ ACD

Fig. 4. 2

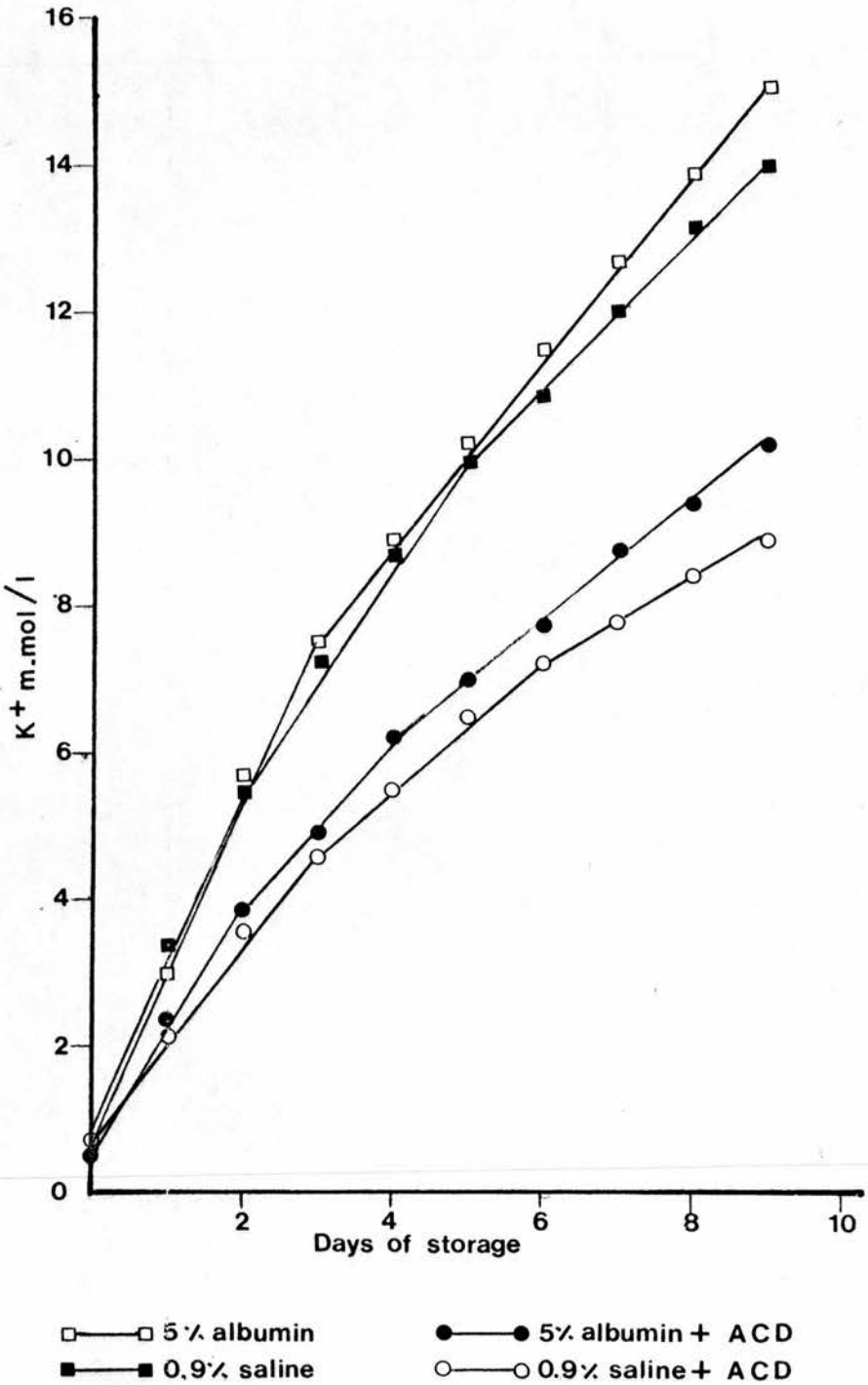

16
14
12
10
8
6
4
2
0
K+ m.mol/l
2
4
6
8
10
Days of storage
5% albumin
0.9% saline
5% albumin + ACD
0.9% saline + ACD

TABLE 4.1

Hb concentration (mg/dl) in the supernatant of frozen red cells processed by the IBM 2991 and resuspended in isotonic saline

	DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	DAY 8	DAY 9	pH
1.	400	700	760	770	780	840	870	890	970	1020	6.83
2.	390	730	770	780	790	850	870	880	940	980	6.88
3.	240	450	460	470	490	530	580	580	630	670	6.7
4.	340	530	690	720	720	720	750	760	870	870	6.71
5.	410	660	870	910	940	970	1000	1030	1100	1130	6.77
6.	390	640	810	850	860	870	870	900	970	1000	6.77
7.	420	670	850	870	880	890	900	900	1000	1040	6.64
8.	300	690	800	840	870	910	1030	1080	1140	1200	6.62
9.	430	740	850	900	910	970	990	1010	1040	1090	6.75
10.	400	770	890	980	970	1030	1050	1070	1110	1140	6.77
Mean	370	650	770	800	820	850	890	910	970	1010	6.75
S.D.	61	99	126	141	139	143	143	153	148	153	0.12

TABLE 4.2

Hb concentration (mg/dl) in the supernatant of frozen red cells processed by the IBM 2991 and resuspended in 5% albumin solution

	DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	DAY 8	DAY 9	pH
1.	280	470	480	480	490	500	510	530	560	600	6.83
2.	290	630	640	640	650	660	670	720	740	780	6.88
3.	200	380	380	380	430	430	460	470	500	520	6.70
4.	290	440	560	560	580	590	610	620	640	700	6.71
5.	390	560	670	680	700	770	820	840	900	950	6.77
6.	340	590	750	770	770	800	800	820	840	940	6.77
7.	360	580	730	740	750	760	790	800	840	900	6.64
8.	180	450	550	640	660	680	710	730	770	800	6.62
9.	220	370	390	400	440	450	460	470	470	500	6.75
10.	160	270	320	340	370	380	400	430	430	470	6.77
Mean	270	470	540	560	580	600	620	640	670	710	6.75
S.D.	79	115	150	155	143	154	157	159	171	185	120

TABLE 4.3

(K^+) level (m mol/l) in the supernatant of frozen red cell processed by the IBM 2991 and resuspended in isotonic saline

	DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	DAY 8	DAY 9
1.	1.0	4	6.25	8.0	10	11.5	12	14	15.5	16
2.	0.5	3.5	5.0	7.0	8.5	10	10.5	12.5	13	13.5
3.	0.5	3.0	4.5	6.0	7.5	8.5	9.5	11.5	12	13
4.	0.5	2.5	5.0	6.5	8.5	9.0	10	10.5	12	12.5
5.	1.0	3.0	5.0	7.0	8.5	9.0	10	11	12	13
6.	0	2.5	5.0	7.0	8	10	11	11	13	14
7.	0.5	5.5	7.0	8.5	10	10.5	11.5	12.5	14	15
8.	0.5	3.5	6.5	8.75	10.5	11.75	12.25	14	14.5	15
Mean	0.56	3.4	5.5	7.3	8.9	10	10.8	12.1	13.25	14
S.D.	0.34	0.98	0.92	0.98	1.09	1.19	1.01	1.36	1.31	1.23
n	8	8	8	8	8	8	8	8	8	8

TABLE 4.4

K^+ level (m mol/l) in the supernatant of frozen red cells processed by the IBM 2991 and resuspended in 5% albumin

	DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	DAY 8	DAY 9
1.	0	3.0	5.5	6.75	8.5	10	11.5	12.5	14	15
2.	0	3.5	6.0	7.5	9.0	11	12	13.5	15	16
3.	0.25	3.5	5.5	7.0	9.75	11	12	15	15.5	19
4.	1.0	3.0	5.75	7.25	8.75	10	11.5	12.25	13.75	15
5.	1.5	3.0	5.75	7.5	9.0	10.25	11.5	12.25	13	14.75
6.	0.25	3.25	7.0	9.25	11	12.75	14.25	15	17.25	19
7.	1.0	3.0	6.25	8.25	10	12	13.5	14	16	17.7
8.	0.5	4.0	7.5	9.5	11.5	12.25	14.5	16	16.5	17
9.	1.0	2.0	3.5	5.0	4.75	5.75	6.25	7.0	7.75	8.25
10.	0.25	2.0	3.25	5.0	6.0	7.0	8.0	9.5	10.25	11.25
Mean	0.57	3	5.6	7.3	8.8	10.2	11.5	12.7	13.9	15.3
S.D.	0.52	0.63	1.34	1.51	2.08	2.24	2.6	2.72	2.94	3.38

TABLE 4.5

Hb concentrations (mg/dl) in the supernatant of frozen red cells processed by the IBM 2991 and resuspended in saline-ACD medium

		DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	DAY 8	DAY 9	pH
1.	4781	60	210	190	250	250	290	320	320	370	370	6.0
2.	4776	80	140	200	180	190	220	240	250	280	300	6.0
3.	31151	80	170	190	230	260	270	280	300	320	330	6.1
4.	31163	80	250	280	330	340	350	370	410	430	430	6.1
5.	31153	80	210	240	250	270	300	300	330	340	340	6.35
6.	31154	150	340	380	400	430	470	480	500	520	540	6.2
7.	5507	200	430	480	490	530	550	560	580	610	610	N.D.
8.	5489	60	180	210	230	250	270	270	290	310	310	N.D.
9.	6589	100	230	260	290	300	310	310	360	380	400	6.0
10.	6573	90	180	210	230	240	260	280	290	310	320	5.93
Mean		98	230	260	280	300	330	340	360	380	390	6.08
S.D.		45	88	95	94	102	103	102	105	105	105	0.19

TABLE 4.6

Hb concentration (mg/dl) in the supernatant of frozen red cells processed by the IBM 2991 and resuspended in ACD-albumin medium

		DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	DAY 8	DAY 9	pH
1.	4781	50	130	150	170	180	220	230	230	270	280	6.0
2.	4776	50	120	120	130	160	160	170	170	190	200	6.0
3.	31151	60	120	120	160	180	190	200	210	210	220	6.1
4.	31163	50	160	180	210	230	230	240	280	290	300	6.1
5.	31153	40	120	150	180	200	200	220	230	250	250	6.35
6.	31154	60	210	240	260	290	330	330	350	370	370	6.2
7.	5507	120	300	330	340	360	380	380	410	430	430	N.D.
8.	5489	40	120	140	160	170	180	200	210	230	230	N.D.
9.	6589	82	150	180	200	210	250	250	260	260	270	6.0
10.	6573	61	130	160	170	180	200	200	210	210	220	5.93
Mean		60	150	170	200	210	230	240	250	270	280	6.08
S.D.		26	58	64	61	63	70	65	73	76	73	19

TABLE 4.7

(K^+) level (m mol/l) in the supernatant of frozen red blood cells processed by the IBM 2991 and resuspended in saline-ACD medium

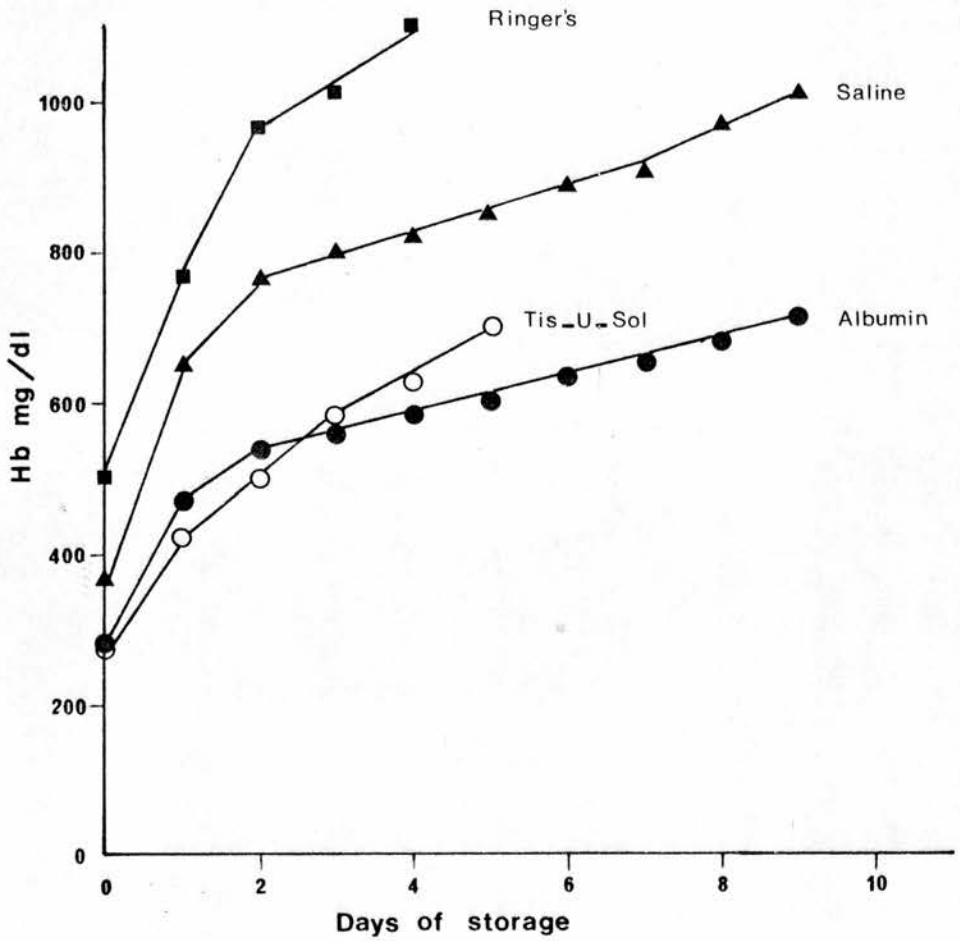
	DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	DAY 8	DAY 9
4781	1	1.5	2.5	3.5	4.0	5.0	5.5	6.0	7.0	7.5
4776	0.5	2.8	4.0	5.0	6.5	7.5	8.1	9.0	9.5	10.5
31151	0.5	2.0	4.0	5.0	6.0	6.5	7.0	8.0	9.0	9.5
31163	0.2	2.0	4.0	4.5	5.5	7.0	8.0	8.0	8.5	8.5
31153	0.5	2.0	3.0	4.0	5.0	6.0	6.5	7.0	7.5	8.0
31154	0.5	2.0	3.5	4.2	5.0	6.5	6.5	7.0	7.5	8.0
5507	0.5	2.0	3.0	4.5	5.5	6.0	7.0	7.5	8.0	8.5
5489	0.5	2.0	3.0	4.0	4.5	5.0	6.5	6.5	7.0	7.5
6589	1.0	2.5	3.5	4.7	5.5	6.0	7.0	7.7	8.0	8.5
6573	0.5	3.5	6.0	6.5	7.5	9.5	10.5	11.5	12	13
Mean	0.57	2.2	3.6	4.6	5.5	6.5	7.2	7.8	8.4	8.9
S.D.	0.26	0.57	0.97	0.82	1.0	1.3	1.36	1.5	1.5	1.69

TABLE 4.8

(K^+) level (m mol/l) in the supernatant of frozen red blood cells processed by the IBM 2991 and resuspended in 5% albumin-ACD medium

	DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	DAY 8	DAY 9
4781	1.2	1.5	3.0	4.0	5.0	6.0	7.0	8.0	8.5	9.5
4776	0.5	3.0	4.5	5.5	7.0	8.0	8.5	9.7	10.5	11.5
31151	0	2.0	4.0	4.5	5.2	6.0	7.0	7.5	7.7	9.0
31163	0	2.5	4.0	5.0	6.0	6.5	6.5	8.7	9.5	10
31153	0.5	2.0	3.5	4.5	5.5	6.5	7.0	8.0	9.0	9.5
31154	0.5	2.5	4.0	4.7	5.2	6.7	7.0	8.5	8.7	9.7
5507	0.5	2.0	3.5	4.5	5.5	6.5	7.5	8.0	8.5	9.5
5489	0.5	2.0	3.0	4.0	5.0	6.0	6.0	7.5	8.0	9.0
6589	1.0	2.5	3.7	5.0	6.0	6.5	7.5	8.2	9.0	9.5
6573	0.75	4.0	6.0	7.5	11.5	12	13	14.5	14.5	15
Mean	0.5	2.4	3.9	4.9	6.2	7.0	7.7	8.8	9.4	10.2
S.D.	0.39	0.7	0.87	1.02	1.96	1.8	1.97	2.08	1.96	1.82

Fig 4.3


Ringer's
Saline
Tis_U_Sol
Albumin
Hb mg/dl
1000
800
600
400
200
0
0
2
4
6
8
10
Days of storage

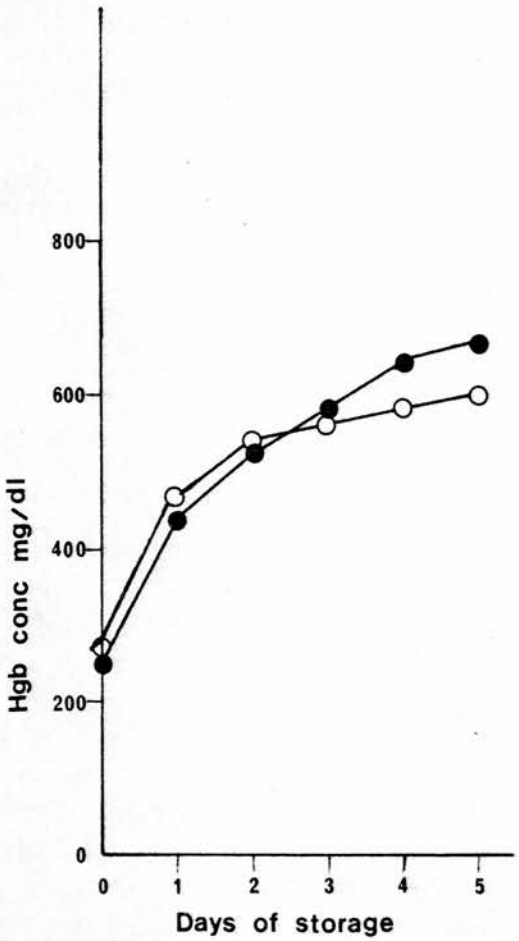


Fig 4.4a

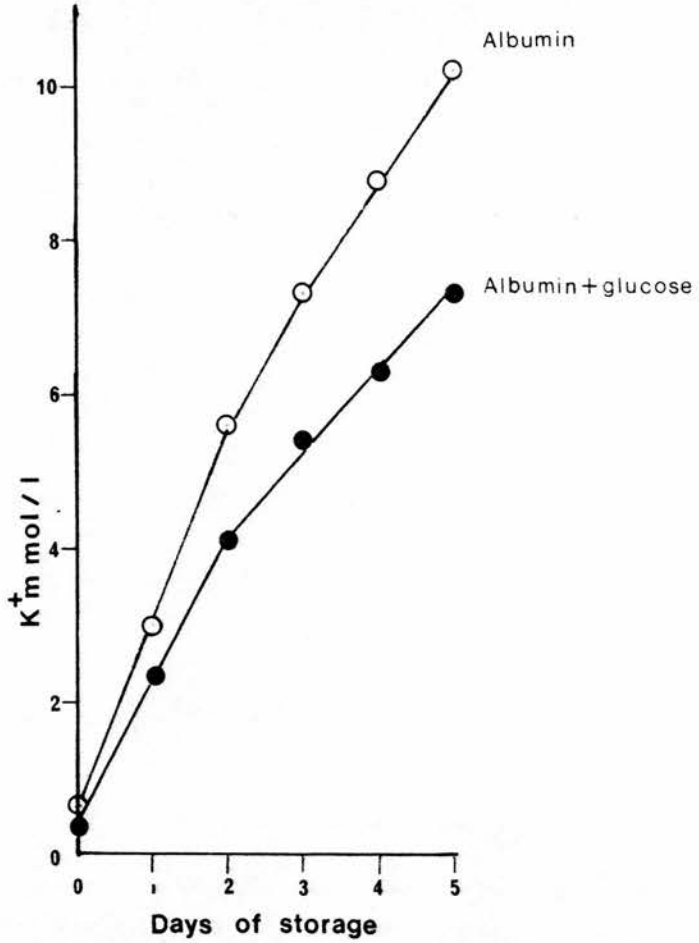


Fig 4.4b

TABLE 4.9

Hb concentration (mg/dl) in the supernatant of previously frozen-thawed-washed erythrocytes after resuspension in isotonic saline at pH 6.1

Unit No.	DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	DAY 8	DAY 9	pH
36104	140	280	350	390	410	420	430	460	480	500	6.1
63036	50	160	200	220	230	250	250	260	280	300	6.1
07413	100	390	480	540	550	550	560	580	600	640	6.1
63042	80	380	460	520	530	550	560	620	630	650	6.0
63037	140	400	510	550	590	600	620	620	650	660	6.25
Mean	102	322	400	444	462	474	484	508	528	550	6.11
S.D.	39	103	127	141	146	142	148	153	154	154	0.09

TABLE 4.10

Hb concentration (mg/dl) in the supernatant of previously frozen-thawed washed erythrocytes after resuspension in isotonic saline at pH 6.5

Unit No.	DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	DAY 8	DAY 9	pH
36099	140	480	630	660	760	760	770	790	830	850	6.4
63038	190	540	680	750	790	810	830	830	850	900	6.47
07365	240	740	930	1000	1060	1080	1110	1150	1190	1250	6.46
36748	220	600	730	830	880	890	900	910	910	960	6.63
63040	120	460	580	620	660	680	680	680	710	710	6.44
Mean	182	564	710	772	830	844	858	872	998	934	6.48
S.D.	51	113	135	151	151	152	162	176	179	199	0.09

TABLE 4.11

Hb concentration (mg/dl) in the supernatant of previously frozen-thawed-washed erythrocytes after resuspension in isotonic saline at pH 6.8

	DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	DAY 8	DAY 9	pH
24939	450	800	890	930	990	1000	1140	1170	1260	1280	6.93
24940	370	740	870	960	1020	1080	1140	1240	1310	1360	6.8
24947	440	780	970	1150	1230	1300	1350	1420	1510	-	6.8
24942	550	970	1100	1440	1470	1500	1580	1630	1700	-	6.8
26753	170	320	400	450	500	530	590	630	700	-	6.8
27661	360	580	700	760	830	850	870	950	1020	-	6.7
27673	190	360	500	590	660	720	770	820	870	-	6.8
26033	180	360	510	690	650	740	700	810	840	-	6.9
Mean	339	614	743	871	919	965	1018	1084	1151	1320	6.82
S.D.	144	245	253	319	325	322	343	341	351	57	0.07

TABLE 4.12

Effect of different resuspension media on the rate of haemolysis as measured by the haemoglobin release (mg/dl of the supernatant) during post-wash storage at +4°C for 9 days. All units processed by the IBM 2991

	n*	DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	DAY 8	DAY 9
1. 0.9% NaCl solution	10	370	650	770	800	820	850	890	910	970	1010
2. 5% albumin in 0.9% NaCl + ACD	10	60	150	170	200	210	230	240	250	270	280
3. 0.9% NaCl + ACD	10	98	230	260	280	300	330	340	360	380	390
4. 5% albumin in 0.9% NaCl	10	270	470	540	560	580	600	620	640	670	710
5. Tis-U-Sol	4	280	423	500	580	625	700	N.D.	N.D.	N.D.	N.D.
6. Ringer's lactate Solution	8	500	770	970	1010	1100	1200	N.D.	N.D.	N.D.	N.D.
Statistics:											
Group (1) Vs (2) t-test		17.405	13.819	16.023	13.821	13.534	13.003	13.082	11.909	13.821	13.851
p		←				< 0.0005					→
Group (1) Vs (3) t-test		14.613	9.883	12.239	10.662	10.151	9.693	9.536	8.926	10.202	10.322
p	10	←				< 0.0005					→
Group (1) Vs (4) t-test		4.325	3.919	4.277	4.205	4.305	4.222	4.357	4.280	5.041	4.513
p	10	←				< 0.005					→

TABLE 4.12 (cont'd.)

		n*	DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	DAY 8	DAY 9
Group (2) Vs (3)	t-test		4.3842	7.0682	7.7954	8.2158	7.0183	7.9505	7.8023	10.5773	11.5743	10.8936
	p	10	<				0.0005					>
Group (2) Vs (4)	t-test		8.5416	9.3655	9.0450	8.5440	9.6759	8.4238	8.6423	8.5727	8.3441	8.5424
	p	10	<				0.0005					>
Group (3) Vs (4)	t-test		7.895	6.573	7.744	6.517	7.142	6.652	6.451	6.091	5.555	6.334
	p	10	<				0.0005					>

TABLE 4.13

Effect of different resuspension media on the rate of potassium release (m mol/l) from the red cells during post-thaw storage at +4°C for nine days. All units processed by the IBM 2991 Automatic Cell Processor

	n*	DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	DAY 8	DAY 9
Gp I 0.9% NaCl Solution	10	0.9	3.9	6.2	7.5	8.9	10.6	10.75	11	11.5	12.5
Gp II 5% albumin + ACD	10	0.5	2.4	3.9	4.9	6.2	7.0	7.7	8.8	9.4	10.2
Gp III 0.9% NaCl + ACD	10	0.57	2.2	3.6	4.6	5.5	6.5	7.2	7.8	8.4	8.9
Gp IV 5% albumin sol.	10	0.8	4.3	6.9	8.8	10.6	12.2	12.75	13.5	14	14.5
Tis-U-Sol	4	6	8.5	10	11.5	12.5	14.5	n.d.	n.d.	n.d.	n.d.
Ringer's lactate	8	4.5	6.25	8	11.25	14.24	18	n.d.	n.d.	n.d.	n.d.
Statictics											
Gp I Vs II t-test		0.104	2.624	3.834	5.127	3.538	3.956	4.080	3.822	4.773	5.018
p		>0.45	<0.05	<0.005	←			<0.0005	→		
Gp I Vs III t - test		0.056	3.290	4.194	6.525	6.986	5.920	6.182	6.190	7.185	7.075
p		>0.47	←			<0.005	→				
Gp I Vs IV t-test		0.060	1.085	0.124	0.071	0.138	0.192	0.671	1.073	0.578	1.025
p		>0.4	>0.15	>0.45	>0.45	>0.45	>0.4	>0.25	>0.15	>0.25	>0.15
Gp II Vs III t-test		0.382	2.279	3.420	2.556	1.750	2.114	1.305	3.453	3.254	5.522
p		>0.35	<0.05	<0.005	<0.05	=0.05	<0.05	>0.1	<0.005	<0.005	<0.0005
Gp II Vs IV t-test		0.137	1.718	2.691	3.311	2.328	2.833	2.986	2.964	3.349	3.560
p		>0.4	>0.05	<0.05	<0.005	<0.05	<0.01	<0.01	<0.01	<0.005	<0.005
Gp III Vs IV t-test		0.028	2.418	2.783	4.083	3.924	3.886	3.892	4.227	4.590	4.744
p		>0.48	<0.05	<0.05	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005

Fig. 4.5

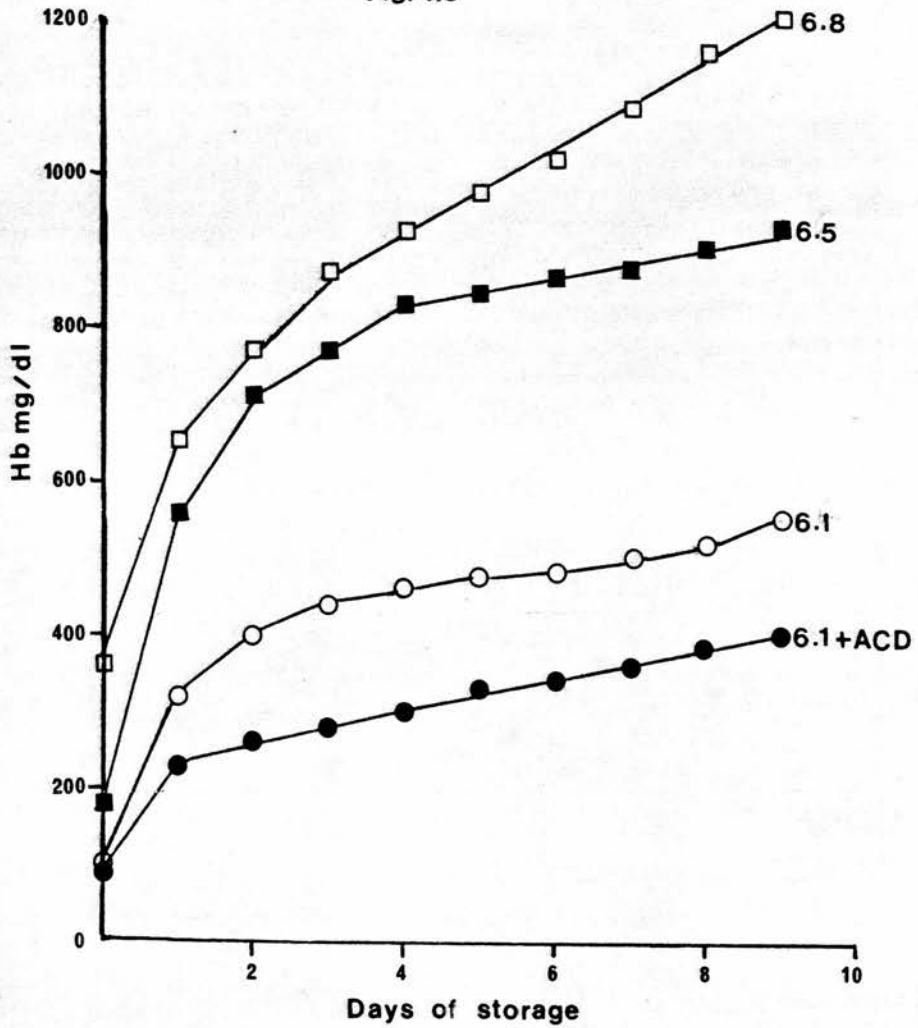

1200
1000
800
600
400
200
0
Hb mg/dl
2
4
6
8
10
Days of storage
6.8
6.5
6.1
6.1+ACD

TABLE 4.14

Hb concentration (mg/dl in the supernatant of frozen-thawed-washed erythrocytes resuspended in isotonic saline with different pH values

		DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	DAY 8	DAY 9	pH
Gp I Cells resuspended in ACD-Saline at pH6.1		98	230	260	280	300	330	340	360	380	390	6.08
GpII cells resuspended in saline at pH 6.1		100	320	400	440	460	475	480	500	520	550	6.11
Gp III cell resuspended in saline at pH 6.5		180	580	710	770	830	840	860	870	900	930	6.5
GpIV cells resuspended in saline at pH 6.8		340	610	740	870	920	970	1010	1080	1150	1320	6.82
Statistics:	Comparison	between all groups										
Gp I Vs II	t-test	0.542	1.732	2.339	2.575	2.424	2.275	2.293	2.176	2.108	2.010	
	p	>0.3	>0.05	←	←	←	<0.05	→	→	→	→	
Gp I Vs III	t-test	3.314	6.256	7.461	7.699	8.019	7.810	7.622	7.104	7.056	6.997	
	p	<0.025		←	←	←	<0.0025	→	→	→	→	
Gp I Vs IV	t-test	5.046	4.569	5.535	5.520	5.662	5.917	5.956	6.371	6.566	11.856	
	p	←	←	←	←	←	<0.005	→	→	→	→	
Gp II Vs III	t-test	2.169	4.210	4.207	4.092	4.300	4.307	4.006	3.796	3.767	3.696	
	p	<0.05	←	←	←	←	<0.01	→	→	→	→	
Gp II Vs IV	t-test	3.550	2.493	2.779	2.790	2.928	3.181	3.251	3.520	3.705	6.560	
	p	<0.0125	←	<0.05	→	→	←	←	<0.025	→	→	
Gp III Vs IV	t-test	2.316	0.421	0.262	0.643	0.567	0.778	0.963	1.274	1.480	2.562	
		<0.05	>0.3	>0.4	>0.25	>0.3	>0.25	>0.2	>0.1	>0.05	<0.05	

Fig.4.6

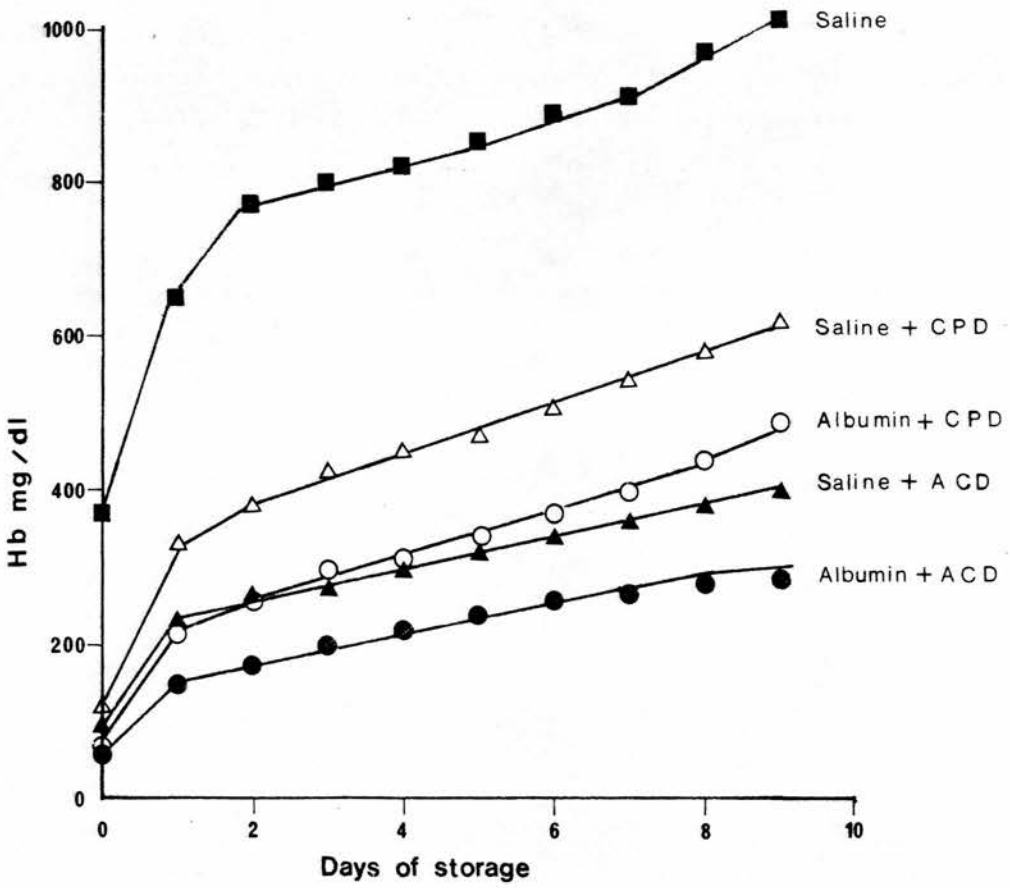

1000
800
600
400
200
0
Hb mg/dl
0
2
4
6
8
10
Days of storage
Saline
Saline + CPD
Albumin + CPD
Saline + ACD
Albumin + ACD

TABLE 4.15

Hb concentration (mg/dl) in the supernatant of frozen-thawed-washed erythrocytes resuspended in saline - CPD medium

Unit No.	DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	DAY 8	DAY 9	pH
26025	170	440	500	560	600	630	650	650	710	750	6.61
26028	115	340	390	430	460	470	490	500	570	610	6.68
31176	60	180	220	230	250	260	270	280	300	340	6.67
26024	110	370	430	470	490	520	540	540	670	700	6.66
31171	120	360	400	440	480	480	500	510	600	650	6.73
Mean	115	338	388	426	456	472	490	496	570	610	6.67
S.D.	39	96	103	121	127	134	138	135	161	160	0.04

TABLE 4.16

Hb concentration in the supernatant of frozen-thawed-washed erythrocytes resuspended in albumin-CPD medium

Unit No.	DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	DAY 8	DAY 9	pH
26025	100	290	340	390	410	430	460	460	530	600	6.61
26028	70	210	260	300	310	320	350	350	400	450	6.68
31176	50	110	150	160	170	180	190	190	210	250	6.67
26024	60	230	280	320	340	340	390	390	450	500	6.66
31171	70	220	270	300	310	330	340	350	400	450	6.73
Mean	70	212	260	294	308	320	346	348	398	450	6.67
S.D.	19	65	69	84	87	90	99	99	118	127	0.04

TABLE 4.17

Hb concentration in the supernatant (mg/dl) of frozen red cells processed by the manual method and resuspended in isotonic saline

	Unit No.	DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	DAY 8	DAY 9
1.	1965	230	440	530	620	640	730	N.D.	N.D.	N.D.	N.D.
2.	1969	160	330	470	550	600	770	"	"	"	"
3.	1962	160	330	400	500	520	1070	"	"	"	"
4.	1960	110	180	200	230	260	260	"	"	"	"
5.	24136	470	710	800	840	870	900	"	"	"	"
6.	24137	200	360	420	490	530	550	"	"	"	"
7.	24142	310	570	620	680	700	710	"	"	"	"
8.	24139	290	520	610	700	710	710	"	"	"	"
9.	34937	210	360	440	470	590	560	640	750	770	800
10.	6442	250	560	590	710	780	810	900	990	1020	1100
	Mean	240	430	500	580	620	700	770	870	900	950
	S.D.	102	146	153	161	159	207	196	183	190	223

TABLE 4.18

Hb concentration in the supernatant (mg/dl) of frozen red blood cells processed by the manual method and resuspended in 5% albumin solution

	Unit No.	DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	DAY 8	DAY 9
1.	1965	200	440	550	630	630	1380	N.D.	N.D.	N.D.	N.D.
2.	1969	130	240	330	370	470	500	"	"	"	"
3.	1962	90	200	250	300	300	360	"	"	"	"
4.	1960	160	330	410	450	480	510	"	"	"	"
5.	24136	150	600	720	770	770	780	"	"	"	"
6.	24137	170	310	360	430	450	450	"	"	"	"
7.	24142	220	420	490	530	540	540	"	"	"	"
8.	24139	150	420	500	550	570	570	"	"	"	"
9.	34933	170	320	360	400	470	470	520	530	580	600
10.	6442	210	460	480	560	670	670	740	770	800	850
	Mean	160	330	440	500	530	620	630	650	690	720
	S.D.	40	123	126	138	132	290	170	183	170	190

TABLE 4.19

(K^+) levels in the supernatant (m mol/l) of frozen red blood cells processed by the manual method and resuspended in isotonic saline

Unit No.	DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	DAY 8	DAY 9
1965	1.0	3.0	5.0	7.0	8.0	11	N.D.	N.D.	N.D.	N.D.
1969	0.5	6.0	9.0	10	11.7	13.5	"	"	"	"
1962	1.0	3.0	5.0	7.0	8.0	11	"	"	"	"
1960	0	2.0	4.0	5.0	6.5	7.5	"	"	"	"
24136	2.0	5.0	7.0	8.5	9.5	10.7	"	"	"	"
24137	0.5	3.5	5.5	7.0	8.0	9.0	"	"	"	"
24142	2.0	6.0	9.5	11.2	13	14.5	"	"	"	"
24139	1.5	4.5	7.0	8.0	9.5	10.7	"	"	"	"
34937	0.5	2.5	4.5	5.5	7.5	9.0	9.5	10.5	11.	12
6442	0.5	3.5	6.0	6.5	7.5	9.5	10.5	11.5	12	13
Mean	0.9	3.9	6.2	7.5	8.9	10.6	10	11	11.5	12.5
S.D.	0.69	1.4	1.86	1.92	2.04	2.1	1.0	1.0	1.0	1.0

TABLE 4.20

(K^+) level in the supernatant (m mol/l) of frozen red blood cells processed by the manual method and resuspended in 5% albumin solution

Unit No.	DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	DAY 8	DAY 9
1965	1.0	4.0	7.0	9.5	11	12.2	N.D.	N.D.	N.D.	N.D.
1969	0.5	3.5	6.0	8.5	10	13.7	N.D.	N.D.	N.D.	N.D.
1962	0.5	2.5	4.5	6.5	7.5	11	"	"	"	"
1960	0.5	2.5	4.5	6.5	8.0	9.0	"	"	"	"
24136	0.5	6.0	9.0	11	12.2	13.5	"	"	"	"
24137	1.0	4.5	7.0	8.0	10	11	"	"	"	"
24142	2.0	7.0	11	13.7	15	17	"	"	"	"
24139	1.0	6.0	9.0	11	12	13.5	"	"	"	"
34933	0.5	3.0	5.0	6.0	9.0	10	11	12.5	13.5	14
6442	0.75	4.0	6.0	7.5	11.5	12	13	14.5	14.5	15
Mean	0.8	4.3	6.9	8.8	10.6	12.2	12	13.5	14	14.5
S.D.	0.48	1.57	2.17	2.47	2.22	2.27	1.58	1.68	1.0	1.0

TABLE 4.21

Hb concentration in the supernatant (mg/dl) of frozen red blood cells processed by the manual method and resuspended in saline - ACD medium

Unit No.	DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	DAY 8	DAY 9	pH
34950	86	180	210	230	240	250	280	290	290	300	5.93
5695	370	460	480	510	510	510	550	550	540	550	5.92
34947	230	450	480	470	500	540	580	630	630	640	6.1
62101	180	280	310	340	360	370	410	430	430	430	5.94
6432	200	310	340	360	400	420	430	460	460	490	6.01
6444	300	530	580	600	650	650	650	650	N.D.	N.D.	6.15
6446	80	250	280	310	350	380	400	440	460	480	6.0
34956	120	300	320	340	370	400	420	450	460	490	6.39
62075	100	350	390	450	480	500	520	540	560	580	6.05
5698	160	360	380	410	430	460	480	500	530	570	6.0
Mean	180	340	370	400	430	450	470	490	490	500	6.05
S.D.	96	107	110	108	113	110	107	105	97	99	0.14

TABLE 4.22

Hb concentration in the supernatant (mg/dl) of frozen red blood cells processed by the manual method and resuspended in 5% albumin - ACD medium

	Unit No.	DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	DAY 8	DAY 9
1.	34950	60	110	130	150	150	160	170	200	200	210
2.	5695	200	300	340	350	360	360	370	380	390	400
3.	34947	140	300	310	320	350	380	400	420	430	440
4.	62101	140	180	200	230	240	250	260	300	300	310
5.	6432	110	200	200	210	270	270	270	300	300	300
6.	6444	210	350	420	440	450	450	450	470	N.D.	N.D.
7.	6446	80	140	160	190	200	210	220	250	280	300
8.	34956	80	160	180	190	200	210	250	280	320	360
9.	62075	70	160	170	180	210	240	270	300	320	340
10.	5698	120	300	310	320	340	380	400	420	440	450
	Mean	120	220	240	260	280	290	300	330	330	340
	S.D.	53	84	96	94	94	95	92	86	77	77

TABLE 4.23

(K^+) level in the supernatant (m mol/l) of frozen red blood cells processed by the manual method and resuspended in saline -ACD medium

	Unit No.	DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	DAY 8	DAY 9
1.	34950	1.0	3.5	4.75	5.0	7.75	8.0	9.0	10	11	11.5
2.	5695	2.0	3.5	5.0	6.0	7.0	8.0	9.0	9.5	10.5	11
3.	34947	0.5	2.5	4.0	4.5	6.5	7.0	8.0	8.5	9.0	9.5
4.	62101	1.75	4.0	5.0	6.5	8.0	8.75	9.4	10	11.25	11.75
5.	6432	0.5	2.0	3.0	4.0	5.0	6.0	6.5	7.5	8.0	8.5
6.	6444	1.0	3.0	4.25	5.25	6.0	8.0	11	N.D.	N.D.	N.D.
7.	6446	0.5	2.0	3.0	4.0	4.75	5.5	6.0	7.0	7.25	8.0
8.	34956	1.0	2.0	3.5	4.0	4.75	5.25	6.0	6.5	7.0	8.0
9.	62075	0.5	2.0	3.0	3.75	4.5	6.0	6.25	7.0	8.0	8.5
10.	5698	0.5	2.5	3.5	4.5	6.0	7.0	8.0	8.5	9.0	9.5
	Mean	0.925	2.70	3.90	4.75	6.0	6.9	7.9	8.2	9.0	9.6
	S.D.	0.56	0.76	0.82	0.93	1.28	1.22	1.7	1.27	1.6	1.49

TABLE 4.24

(K^+) level in the supernatant (m mol/l) of frozen red blood cells processed by the manual method and resuspended in 5% albumin -ACD medium

	Unit No.	DAY 0	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	DAY 8	DAY 9
1.	34950	1.0	3.0	4.0	5.5	7.5	9.0	9.5	10.25	10.5	11.5
2.	5695	2.0	4.0	5.5	7.0	8.25	9.5	10	10.5	11.5	12
3.	34947	0.5	2.5	3.5	5.0	6.5	8.0	8.5	9.75	10.75	11
4.	62101	1.5	4.0	5.0	6.5	8.5	9.25	10	10.75	12.25	12.25
5.	6432	0.25	2.0	3.0	4.0	5.5	6.0	7.0	8.0	8.5	9.0
6.	6444	1.0	3.0	4.0	6.0	7.0	8.5	11.75	N.D.	N.D.	N.D.
7.	6446	0.75	2.0	3.75	4.25	5.75	6.0	7.0	7.75	8.5	9.0
8.	34956	1.0	2.0	3.5	4.0	5.0	5.75	6.25	7.0	7.5	8.5
9.	62075	0.5	2.0	3.0	4.0	5.0	6.0	7.0	8.0	8.75	9.5
10.	5698	0.75	2.5	4.0	5.0	6.0	7.5	8.5	9.5	10	10.5
	Mean	0.92	2.7	3.9	5.1	6.5	7.5	8.5	9.0	9.8	10.3
	S.D.	0.52	0.79	0.8	1.1	1.27	1.5	1.76	1.44	1.58	1.41

TABLE 4.25

Effect of freezing, thawing, processing and resuspension in saline - ACD medium on the haematological and biochemical parameters of the red cells

	Unit No.	Pre-freezing					Post-Processing					
		2,3 DPG (µ mole/ g Hb)	ATP (µ mole/ g Hb)	W.B.C. (x 10^6/ g Hb	MCV (fl)	storage at 4°C (days)	2.3 DPG (µmole/ g Hb)	ATP (µmole/ g Hb)	W.B.C. (x 10^6/ g Hb)	MCV (fl)	storage in the frozen state (days)	pH
1.	24825	5.1	2.8	40.3	92	2	3.0	2.5	3.7	94	48	6.3
2.	60560	3.5	6.5	33	93	5	2.0	2.8	6.1	98	56	6.0
3.	23623	9.0	4.0	49	89	4	1.8	4.0	4.7	92	56	6.2
4.	23657	5.8	3.5	45	90	4	3.2	3.0	5.9	95	56	6.2
5.	02377	7.8	2.7	50	98	2	7.0	2.5	7.7	101	48	6.2
6.	60767	3.7	2.5	30.8	87	3	3.3	2.2	5.7	90	50	6.2
7.	60844	6.8	2.8	40.5	87	2	5.9	2.8	6.8	89	48	6.0
8.	23650	6.2	3.1	43	89	4	5.5	3.0	7.7	91	56	6.0
9.	01897	7.8	3.0	52.4	91	4	4.6	2.9	13.4	90	59	6.2
10.	02492	N.D.	N.D.	N.D.	N.D.	3	6.8	4.2	5.0	99	45	6.3
11.	01892	11	1.5	53.7	90	4	7.3	2.5	3.0	92	59	6.4
12.	02505	N.D.	N.D.	N.D.	N.D.	2	6.2	2.9	6.2	81	45	6.1
13.	01854	6	1.4	15.6	84	4	2.8	2.5	3.0	85	59	6.0
14.	01896	6.2	2.7	34.6	94	4	3.6	4.0	5.6	93	59	6.2

TABLE 4.25 (cont'd.)

	Unit No.	Pre-freezing					Post-Processing					
		2,3 DPG (μ mole/g Hb)	ATP (μ mole/g Hb)	W.B.C. (x 10^6/g Hb)	MCV (fl)	storage at 4°C (days)	2,3 DPG (μmole/g Hb)	ATP (μmole/g Hb)	W.B.C. (x 10^6/g Hb)	MCV (fl)	storage in the frozen state (days)	pH
15.	60609	2.0	3.8	61	88	5	1.8	3.8	5.5	93	53	6.4
16.	01908	3.1	3.1	49.8	94	4	2.7	2.6	4.0	95	59	6.0
17.	01876	7.6	2.3	60.4	93	4	5.5	2.5	3.8	96	52	6.1
18.	24242	11.8	2.3	39.9	85	2	7.3	N.D.	9.9	86	45	N.D.
19.	02543	N.D.	N.D.	N.D.	N.D.	5	3.3	3.4	10.2	96	66	6.0
20.	01893	5.5	2.5	69.2	73	4	3.3	2.2	8.3	75	53	5.9
21.	61060	7.9	2.9	51	95	3	7.6	2.6	5.0	98	35	6.0
22.	60857	4.8	2.6	64.5	86	2	2.4	2.7	5.6	88	41	6.1
23.	24223	11.5	2.6	68.2	85	2	6.0	1.8	3.0	86	47	6.1
24.	60583	1.3	2.9	44	92	5	0	2.4	4.4	94	49	6.1
25.	24028	4.0	2.7	41	89	2	3.0	2.0	5.2	91	51	N.D.
26.	24158	9.1	3.5	80.8	86	2	6.5	3.1	0	87	45	N.D.
27.	60624	2.7	N.D.	17.4	92	2	1.3	2.5	3.1	95	44	N.D.
28.	60871	7.0	2.7	21.1	91	2	3.9	2.2	5.0	94	51	N.D.
29.	01887	10.6	3.2	10.0	98	4	12.7	2.3	9.6	98	51	N.D.
30.	24845	6.1	2.7	38.1	94	2	5.8	2.5	6.0	91	40	N.D.
31.	01855	5.5	3.0	24.6	90	4	3.8	2.8	4.0	93	51	N.D.
32.	60581	1.5	2.7	46	92	5	2.5	2.8	4.0	94	45	6.0

TABLE 4.25 (cont'd.)

	Unit No.	Pre-freezing					Post-Processing					
		2.3 DPG (µmole/g Hb)	ATP (µmole/g Hb)	W.B.C. (x 10^6 g Hb)	MCV (fl)	Storage at 4°C (days)	2,3 DPG (µmole/g Hb)	ATP (µmole/g Hb)	W.B.C. (x 10^6 g Hb)	MCV (fl)	Storage in the frozen state (days)	pH
33.	60768	4.7	2.4	41.8	90	3	6.0	2.1	3.5	92	38	6.4
34.	01886	9.5	2.4	73	89	4	7.3	2.3	3.4	92	48	6.3
35.	24236	14	2.5	63.3	98	2	7.3	2.3	6.3	101	42	6.1
36.	24195	12	2.9	61.2	90	2	11.3	2.9	10	91	42	6.0
37.	24205	12.7	3.0	59.9	91	2	10.4	2.9	5.2	91	42	6.1
38.	23647	7.2	2.9	68	96	4	5.9	2.2	2.1	96	42	6.0
39.	02262	11.7	3.2	85.7	86	2	9.6	2.9	14.4	87	42	6.2
40.	24251	13.7	3.2	61.9	92	2	7.6	3.2	2.8	94	41	6.1
41.	60785	6.0	2.8	23	92	3	5.9	2.6	3.4	96	37	6.3
42.	02215	7.0	2.8	52.8	90	2	6.7	2.8	11	91	40	6.3
43.	24238	12.7	3.2	39.1	87	2	8.2	2.9	4.5	89	41	6.3
44.	24041	4.6	2.9	55	89	2	1.4	2.8	3.2	91	42	6.2
45.	24040	3.1	2.5	104	96	2	1.7	2.4	19.2	98	42	6.4
46.	02224	8.9	2.9	38.1	89	2	6.3	2.7	10.8	116	40	6.0
47.	24209	12	2.5	118	100	2	7.5	4.0	N.D.	N.D.	41	6.3
48.	60779	4.4	2.4	42.2	91	3	5.6	2.0	3.3	94	65	6.3
49.	02502	N.D.	N.D.	N.D.	N.D.	2	7.0	2.4	4.1	95	61	6.4

TABLE 4.25 (cont'd.)

	Unit No.	Pre-freezing					Post-processing					pH
		2,3 DPG (μmole/g Hb)	ATP (μmole/g Hb)	W.B.C. (x 10^6/g Hb)	MCV (fl)	storage at 4°C (days)	2,3 DPG (μmole/g Hb)	ATP (μmole/g Hb)	W.B.C. (x 10^6 g Hb)	MCV (fl)	storage in the frozen state (days)	
50.	02271	9.6	N.D.	56.2	90	2	7.2	2.8	5.5	89	68	6.2
51.	02267	10.3	4.3	39.6	79	2	10.8	3.7	2.5	91	68	5.9
52.	02265	7.7	2.3	54.7	88	2	5.2	2.0	19.2	89	68	6.1
53.	60770	6.5	2.7	37	84	3	4.5	2.3	5.8	88	65	6.1
54.	24027	1.6	3.0	45	85	2	0.8	2.9	9.1	87	70	6.3
55.	61048	9.4	3.2	34	96	3	11.8	2.4	2.5	98	58	6.3
56.	24758	6.5	2.7	37	84	3	4.6	2.6	5.7	96	34	6.2
57.	02248	7.7	3.3	62	96	2	5.0	2.5	9.7	95	38	6.2
58.	02263	12	4.1	62.2	93	2	7.1	3.0	0	94	38	6.0
59.	24197	11.8	3.2	64.4	92	2	7.9	3.3	0	97	39	6.1
60.	24025	1.5	2.8	59	92	2	1.3	2.8	5.5	94	40	6.0
61.	60661	8.6	4.7	29.2	89	2	5.3	3.0	0	90	38	6.0
62.	24042	4.6	3.7	24	95	2	3.0	3.8	10.9	97	40	6.3
63.	02546	N.D.	N.D.	N.D.	N.D.	2	3.0	2.7	5.1	94	27	6.2
64.	60769	5.7	3.4	57.2	91	3	5.0	2.7	8.7	97	30	6.4
65.	23645	10.9	3.2	38.6	90	4	6.4	2.7	5.9	93	36	6.0
66.	60781	4.3	2.8	33.3	82	3	4.5	2.5	1.8	85	30	6.2

TABLE 4.25 (cont'd.)

	Unit No.	Pre-freezing					Post-processing					
		2,3 DPG (μmole/g Hb)	ATP (μmole/g Hb)	W.B.C. (x 10^6 g Hb)	MCV (fl)	storage at 4°C (days)	2,3 DPG (μmole/g Hb)	ATP (μmole/g Hb)	W.B.C. (x 10^6 g Hb)	MCV (fl)	storage in the frozen state (days)	pH
67.	60748	4.6	3.6	37.8	92	3	3.9	3.7	4.1	97	30	6.2
68.	60793	5.6	3.5	35.6	82	3	5.7	2.8	8.0	86	30	6.3
69.	02508	N.D.	N.D.	N.D.	N.D.	2	4.5	2.1	3.4	92	21	6.3
70.	24814	5.7	2.8	38.6	90	2	4.2	2.2	1.3	91	23	6.3
71.	02531	N.D.	N.D.	N.D.	N.D.	2	N.D.	N.D.	N.D.	N.D.	21	6.0
72.	61062	9.6	2.5	42	92	3	N.D.	N.D.	N.D.	N.D.	30	6.2
73.	02331	7.8	2.7	35.7	93	4	4.5	2.7	6.7	96	30	6.2
74.	60598	0	2.0	60	89	5	0.7	2.4	5.9	91	35	6.1
75.	02504	N.D.	N.D.	N.D.	N.D.	2	5.5	2.6	3.9	92	26	6.3
76.	24216	12.9	2.6	99.5	90	2	6.3	2.2	11.1	91	34	6.2
77.	61047	11.2	2.7	31	93	3	13.6	2.0	3.5	97	23	6.3
78.	02474	N.D.	N.D.	N.D.	N.D.	6	3.8	2.6	3.1	96	26	6.1
79.	02507	N.D.	N.D.	N.D.	N.D.	2	0.9	2.8	3.4	92	20	6.0
80.	24808	7.0	2.0	43	87	2	15.1	N.D.	2.5	88	22	6.2
81.	61040	9.8	1.7	17	96	3	10.7	1.1	3.0	97	17	6.3
82.	61032	9.6	2.0	43	86	3	8.5	2.1	1.7	87	17	6.2
83.	24766	5.5	2.9	82.8	91	2	3.1	2.3	4.2	92	22	6.2

TABLE 4.25 (cont'd.)

	Unit No.	Pre-freezing					Post-processing					
		2,3 DPG (μmole/g Hb)	ATP (μmole/g Hb)	W.B.C. (x 10^6/g Hb)	MCV (fl)	storage at 4°C (days)	2,3 DPG (μmole/g Hb)	ATP (μmole/g Hb)	W.B.C. (x 10^6 g Hb)	MCV (fl)	storage in the frozen state (days)	pH
84.	60596	2.0	2.5	76	91	5	1.3	2.3	2.6	91	30	6.1
85.	24816	2.2	N.D.	73.7	91	2	0	2.0	5.6	91	21	6.1
86.	24822	5.5	3.0	29	91	2	2.9	1.6	2.5	91	21	6.3
87.	02473	N.D.	N.D.	N.D.	N.D.	3	2.4	2.3	1.6	92	19	6.1
88.	61038	9.9	2.1	27	87	3	7.0	2.0	1.3	91	16	6.5
89.	60780	5.7	3.7	37.5	90	3	4.7	3.0	4.0	93	32	6.2
90.	61045	9.9	2.8	25	89	3	11	2.0	4.3	94	25	6.4
91.	61065	10.9	2.7	34	89	3	4.8	2.0	3.2	93	25	6.4
92.	60773	5.5	3.7	52.1	88	3	5.8	3.1	13.5	91	32	6.2
93.	23656	5.7	3.2	42	85	4	6.8	3.2	9.7	86	57	6.0
94.	02495	N.D.	N.D.	N.D.	N.D.	4	13.6	N.D.	6.9	90	47	6.1
95.	01853	8.7	3.6	15.3	89	4	5.5	2.6	4.3	92	61	6.2
96.	24239	N.D.	1.8	49.5	90	2	4.6	1.4	7.3	93	50	6.4
97.	01885	10.1	3.1	75.3	87	4	8.5	2.9	18	90	61	6.3
98.	60647	10.8	3.1	49.2	95	2	10	2.6	8	96	55	6.0
99.	02247	8.9	2.6	33.1	91	2	N.D.	N.D.	N.D.	N.D.	39	N.D.
100.	24758	8.9	5.6	50	92	3	N.D.	N.D.	N.D.	N.D.	34	N.D.
	Mean	7.33	2.95	49.1	90.1	2.9	5.53	2.64	5.97	92.4	42.1	6.175
	S.D.	3.3	0.75	20.19	4.26	1.02	3.16	0.56	4.32	4.84	13.7	0.136
	No.	87	85	88	88	100	96	93	95	94	100	90

TABLE 4.26

Summary and comparison between the red cell characteristics before freezing and after processing

	Duration of storage (days)	Hct	M C V fl	W.B.C. x 10⁷ g Hb	pH	2,3 DPG μ mole/ g Hb	ATP μ mole/ g Hb
Before freezing	2.9 ± 1.02	0.661 ± 0.072	90.1 ± 4.3	49.1 ± 20.2	N.D.	7.33 ± 3.3	2.95 ± 0.75
After processing	42.1 ± 13.7	0.663 ± 0.047	92.5 ± 4.5	4.9 ± 4.3	6.18 ± 0.14	* 5.53 ± 3.16	* 2.64 ± 0.56
Statistics:							
No. of experiments	100	96	88-94	88-95	90	87-96	85-93
t	N.D.	N.D.	4.4307	20.324	N.D.	3.754	3.071
p			< .0005	< 0.0005		< 0.0005	< 0.0005

N.B. * a further 15 units were examined after 10 days storage post-thaw in ACD-saline and found to have a mean level of 2.4 μ mol/g Hb of 2,3 DPG and 0.99 μ mol/g Hb ATP.

BACTERIOLOGY RESULTS

At zero day of processing:- All units were sterile with the exception of four units No.60581, 24236, 33647 and 24040 (Table B .1). Although unit No.60581 showed a positive contamination with coliform organism in the subculture blood agar plate which was kept at room temperature, a colony count performed on nutrient agar using 1 ml aliquots was negative. Also, when the primary and subcultures were repeated there was no sign of bacterial growth. Unit No.24236 showed the presence of contamination with staphylococcus albus (coagulase negative) in the three subculture plates incubated, aerobically at room temperature, aerobically at 37°C and anaerobically at 37°C. However the colony count was negative as well as the repeated culture. Unit No.23647 showed a bacterial growth of staphylococcus albus (coagulase negative) in the plate incubated at room temperature and coliform organisms in the plates incubated aerobically and anaerobically at 37°C. Colony count and repeated cultures were negative. The last unit also showed a staphylococcus albus (coagulase negative) growth in the 37°C aerobic plate, but again the colony count and the repeated cultures were sterile.

After 10 days of storage:- All units were sterile (Table B .1 with the exception of unit No.01854 which was contaminated with coliform bacilli; however the colony count was negative, when the cultures were repeated the following organisms were demonstrated.

i) Coliform bacilli and staphylococcus albus (Coagulase negative) in the plate incubated aerobically at 37°C.

ii) Coliform bacilli and micrococcus tetragenous in the anaerobic plate incubated at 37°C.

TABLE B.1

Results of Bacteriology Testing

		0 - Day			10th Day		
	Unit No.	R.T.	Oxygen 37°C	Anaer. 37°C	R.T.	oxygen 37°C	Anaer. 37°C
1.	61032	-	-	-	-	-	-
2.	60596	-	-	-	-	-	-
3.	24816	-	-	-	-	-	-
4.	61038	-	-	-	-	-	-
5.	02473	-	-	-	-	-	-
6.	24822	-	-	-	-	-	-
7.	02507	-	-	-	-	-	-
8.	24808	-	-	-	-	-	-
9.	02531	-	-	-	-	-	-
10.	61062	-	-	-	-	-	-
11.	24814	-	-	-	-	-	-
12.	02508	-	-	-	-	-	-
13.	24766	-	-	-	-	-	-
14.	61040	-	-	-	-	-	-
15.	24216	-	-	-	-	-	-
16.	60598	-	-	-	-	-	-
17.	02504	-	-	-	-	-	-
18.	02474	-	-	-	-	-	-
19.	61045	-	-	-	-	-	-
20.	61065	-	-	-	-	-	-
21.	61047	-	-	-	-	-	-
22.	02331	-	-	-	-	-	-
23.	60780	-	-	-	-	-	-
24.	60773	-	-	-	-	-	-

TABLE B.1 (cont'd.)

		0 - Day			10th Day		
	Unit No.	R.T.	oxygen 37°C	Anaer. 37°C	R.T.	oxygen 37°C	Anaer 37°C
25.	60781	-	-	-	-	-	-
26.	23645	-	-	-	-	-	-
27.	60793	-	-	-	-	-	-
28.	02546	-	-	-	-	-	-
29.	60748	-	-	-	-	-	-
30.	60769	-	-	-	-	-	-
31.	24205	-	-	-	-	-	-
32.	01886	-	-	-	-	-	-
33.	60581	+ Col.B.	-	-	-	-	-
34.	24195	-	-	-	-	-	-
35.	02262	-	-	-	-	-	-
36.	24236	+ S.Alb.	+ S.Alb.	+ S.Alb.	-	-	-
37.	60768	-	-	-	-	-	-
38.	23647	+ S.Alb.	+ Col.B.	+ Col.B.	-	-	-
39.	01855	-	-	-	-	-	-
40.	61071	-	-	-	-	-	-
41.	24158	-	-	-	-	-	-
42.	24845	-	-	-	-	-	-
43.	60871	-	-	-	-	-	-
44.	01887	-	-	-	-	-	-
45.	60624	-	-	-	-	-	-
46.	24028	-	-	-	-	-	-
47.	02265	-	-	-	-	-	-
48.	60779	-	-	-	-	-	-

TABLE B.1 (cont'd.)

		0 - Day			10th Day		
	Unit No.	R.T.	oxygen 37°C	Anaer. 37°C	R.T.	oxygen 37°C	Anaer 37°C
49.	02502	-	-	-	-	-	-
50.	02267	-	-	-	-	-	-
51.	60770	-	-	-	-	-	-
52.	24027	-	-	-	-	-	-
53.	02271	-	-	-	-	-	-
54.	61048	-	-	-	-	-	-
55.	24025	-	-	-	-	-	-
56.	24758	-	-	-	-	-	-
57.	24197	-	-	-	-	-	-
58.	02247	-	-	-	-	-	-
59.	02248	-	-	-	-	-	-
60.	02263	-	-	-	-	-	-
61.	24042	-	-	-	-	-	-
62.	60661	-	-	-	-	-	-
63.	02215	-	-	-	-	-	-
64.	02224	-	-	-	-	-	-
65.	24251	-	-	-	-	-	-
66.	24041	-	-	-	-	-	-
67.	24209	-	-	-	-	-	-
68.	60785	-	-	-	-	-	-
69.	24238	-	-	-	-	-	-
70.	24040	-	+ S.AlB.	-	-	-	-
71.	60583	-	-	-	-	-	-
72.	24223	-	-	-	-	-	-

TABLE B.1 (cont'd.)

		0 - day			10th Day		
	Unit No.	R.T.	oxygen 37°C	Anaer. 37°C	R.T.	oxygen 37°C	Anaer. 37°C
73.	01876	-	-	-	-	-	-
74.	02543	-	-	-	-	-	-
75.	24242	-	-	-	-	-	-
76.	01893	-	-	-	-	-	-
77.	61060	-	-	-	-	-	-
78.	60587	-	-	-	-	-	-
79.	60560	-	-	-	-	-	-
80.	23657	-	-	-	-	-	-
81.	24825	-	-	-	-	-	-
82.	23623	-	-	-	-	-	-
83.	23650	-	-	-	-	-	-
84.	60844	-	-	-	-	-	-
85.	60767	-	-	-	-	-	-
86.	02877	-	-	-	-	-	-
87.	02492	-	-	-	-	-	-
88.	60609	-	-	-	-	-	-
89.	01896	-	-	-	-	-	-
90.	01854	-	-	-	-	+ Col.B.	+ Col.B.
91.	02505	-	-	-	-	-	-
92.	01897	-	-	-	-	-	-
93.	01892	-	-	-	-	-	-
94.	01908	-	-	-	-	-	-
95.	23656	-	-	-	-	-	-

TABLE B.1 (cont'd.)

		0 - Day			10th Day		
	Unit No.	R.T.	oxygen 37°C	Anaer 37°C	R.T.	oxygen 37°C	Anaer 37°C
96.	60647	-	-	-	-	-	-
97.	02495	-	-	-	-	-	-
98.	01853	-	-	-	-	-	-
99.	24239	-	-	-	-	-	-
100.	01885	-	-	-	-	-	-

R.T. = room temperature

Anaer. = Anaerobic

\- = negative growth

Col. B = Coliform bacilli

S.Alb. = Staphylococcus albus

IN VIVO SURVIVAL OF RED CELLS PRESERVED BY LOW-GLYCEROL RAPID FREEZE-THAW TECHNIQUE IN ALUMINIUM CANS

The ultimate criterion of success of any method of red cell preservation is that the recovered cells should survive following transfusion for a normal period. However, many investigators have shown that cells which survive the first 24-hours after transfusion will continue for a normal life span.

Because of the difficulties of accurate estimation of the blood volume in diseased patients and in order to exclude factors of in vivo red cell destruction other than the damage produced by the method of processing, autologous post-transfusion survival was preferred over the homologous tests.

We have investigated the autologous 24-hour post-transfusion survival of frozen red cells that were processed by either the manual or the automatic methods. In addition we have estimated the $T\frac{1}{2}$ ^{51}Cr for red cells processed by the automatic technique and transfused immediately after processing and those resuspended in saline-ACD medium and stored for a further five days, post-processing, at $+4^{\circ}C$ then transfused to the original donors.

The results of the study on the in vivo survival of five autologous units of frozen blood that were processed by the manual technique and transfused on the day of processing are shown in Table 5.1. The mean value for the 24-hour post-transfusion survival was $93.0\% \pm 6.2$ (n=5).

The mean 24-hour post-transfusion survival of autologous frozen blood processed by the automatic method and transfused immediately after washing was $95.0\% \pm 4.3$, n = 5 Table 5.2. The in vivo survival of these cells was also followed over a period of four weeks, the

results shown in Table 5.2 represent the percentage ^{51}Cr survival (not corrected for elution) in each of the four weeks for the five autologous transfusions. The mean survival was 76.0 $\pm$ 9.6, 64 $\pm$ 9.0, 56 $\pm$ 10.5 and 48 $\pm$ 7.5 after one, two, three and four weeks respectively. The $T_{\frac{1}{2}}$ ^{51}Cr for each of the five experiments was 19, 26, 39, 21, and 26 days with a mean of 25 days (Fig. 5.1).

In vivo survival of frozen red cells resuspended in saline-ACD and transfused after five days of storage at +4°C

It was obvious that frozen red cells which are resuspended in saline-ACD medium, after washing, remain stable and sterile over a period of nine days of post-thaw storage at +4°C. However, the behaviour of these cells after transfusion was not known. We have studied the invivo survival of these cells in 10 autologous transfusions after five days of post-thaw storage at +4°C. The results of these experiments are shown in Table 5.3. The mean 24-hour post-transfusion survival was 87% $\pm$ 5.7 n = 10. When the survival was followed over a period of four weeks (5 experiments), we found that the mean survival was 73% $\pm$ 3.0, 63% $\pm$ 2.4, 54% $\pm$ 3 and 44% $\pm$ 1.9 after one, two, three and four weeks respectively. The $T_{\frac{1}{2}}$ ^{51}Cr for each experiment was 23, 22, 23, 23 and 25 days with a mean value of 23 days (Fig. 5.2).

TABLE 5.1

Summary of post-transfusion survival of frozen red cells processed manually and transfused on the same day

	Duration of storage at -180°C (days)	Glycerol Conc. used (g %)	Supnt.Hb of transfused blood (mg/d l)	Supnt.K^+ of transfused blood m mol/l	K^+ intracellular m mol/10^{12}/RBC	In Vitro recovery (%)	Leucocyte x 10^9/l		Post-transfusion survival (%) at	
							Pre tx	after tx	30 min	24 hrs.
J.D.C.	19	23	200	0.5	7.95	96.3	7.0	19.1	106	98.5
D.S.P.	7	24.1	280	1.0	10.2	96.1	6.9	12.9	108	86
D.C.	56	24	270	1.0	8.8	93.9	N.D.	N.D.	88.9	87.6
W.L.	23	24	290	1.0	9.0	96.5	N.D.	N.D.	99	99
K.A.A.	47	22.5	220	0.5	7.8	94	N.D.	N.D.	96.8	95.8
Mean		23.52	252	0.8	8.75	95.36			99.7	93.4
S.D.		0.725	39.62	0.27	0.96	1.3			7.65	6.15

tx = transfusion

TABLE 5.2

Summary of post-transfusion survival of frozen red cells processed by the IBM and transfused on the same day

	Leucocyte count x 10^9/l			Platelet count x 10^9/l			Plasma Haemoglobin				Post-transfusion survival (%) at					
	Pre tx	30min after	24-hr after	Pre tx	30min after	24-hr after	Pre tx g/l	trans-fused blood g/unit	Expec-ted g/l	obser-ved after 30min g/l	30min	24-hr	(1) wk.	(2) wks	(3) wks	(4) wks
C.D.	T% 6.1 N% 58 L% 35 M% 6 E% 1	10.5 76 17 6 1	7.7 66 28 4 2	155	124	155	0.033	2.159	0.70	0.236	104	95.8	74	66	58	49
J.D.C.	R 6.4 N 64 L 31 M 3 E 2	16.7 86 14 0 0	5.6 76 16 5 3	200	185	225	0.031	2.387	0.744	0.272	97.5	92.3	66.7	61	49.3	41.4
M.C.	T 8.0 N 77 L 15 M 8 E 0	24.2 77 18 3 2	9.1 57 38 5 0	170	165	255	0.221	0.854	0.385	0.371	99	101	91	77	73	59

TABLE 5.2 (cont'd.)

	Pre tx	30min after	24-hr after	Pre tx	30min after	24-hr after	Pre tx g/l	trans-fused blood g/unit	Expec-ted g/l	obser-ved after 30min g/l	30min	24-hr	(1) wk.	(2) wks	(3) wks	(4) wks
A.E.R.	T5.3	10.3	4.5	154	150	190	0.035	0.263	0.128	0.118	101	97	80	63	55	51
	N.D.	N.D.	73													
	N.D.	N.D.	24													
	N.D.	N.D.	3													
	N.D.	N.D.	0													
D.B.	T6.5	12	6.5	165	160	180	0.019	0.988	0.366	0.301	95	90	70	52	46	41
	N 61	74	55													
	L 37	22	40													
	M 1	3	4													
	E 1	1	1													
Mean	6.46	14.7	6.7	169	157	201	0.068	1.33	0.465	0.260	99.3	95.2	76.3	63.8	56.3	48.3
S.D.	0.98	5.9	1.8	19	22	39	0.086	0.91	0.256	0.092	3.4	4.3	9.6	9.0	10.5	7.5

Fig 5.1

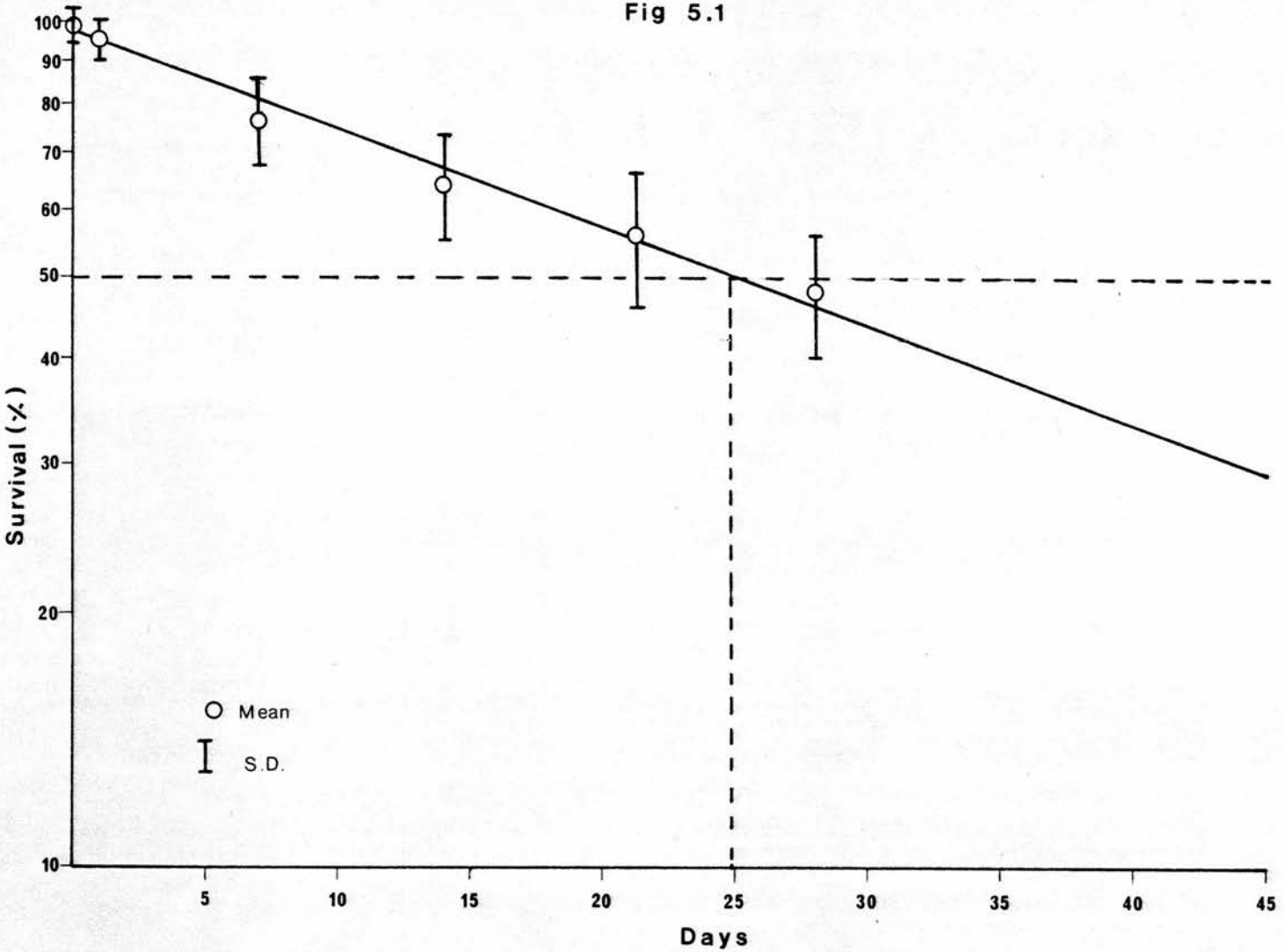

100
90
80
70
60
50
40
30
20
10
Survival (%)
O Mean
I S.D.
5
10
15
20
25
30
35
40
45
Days

TABLE 5.3

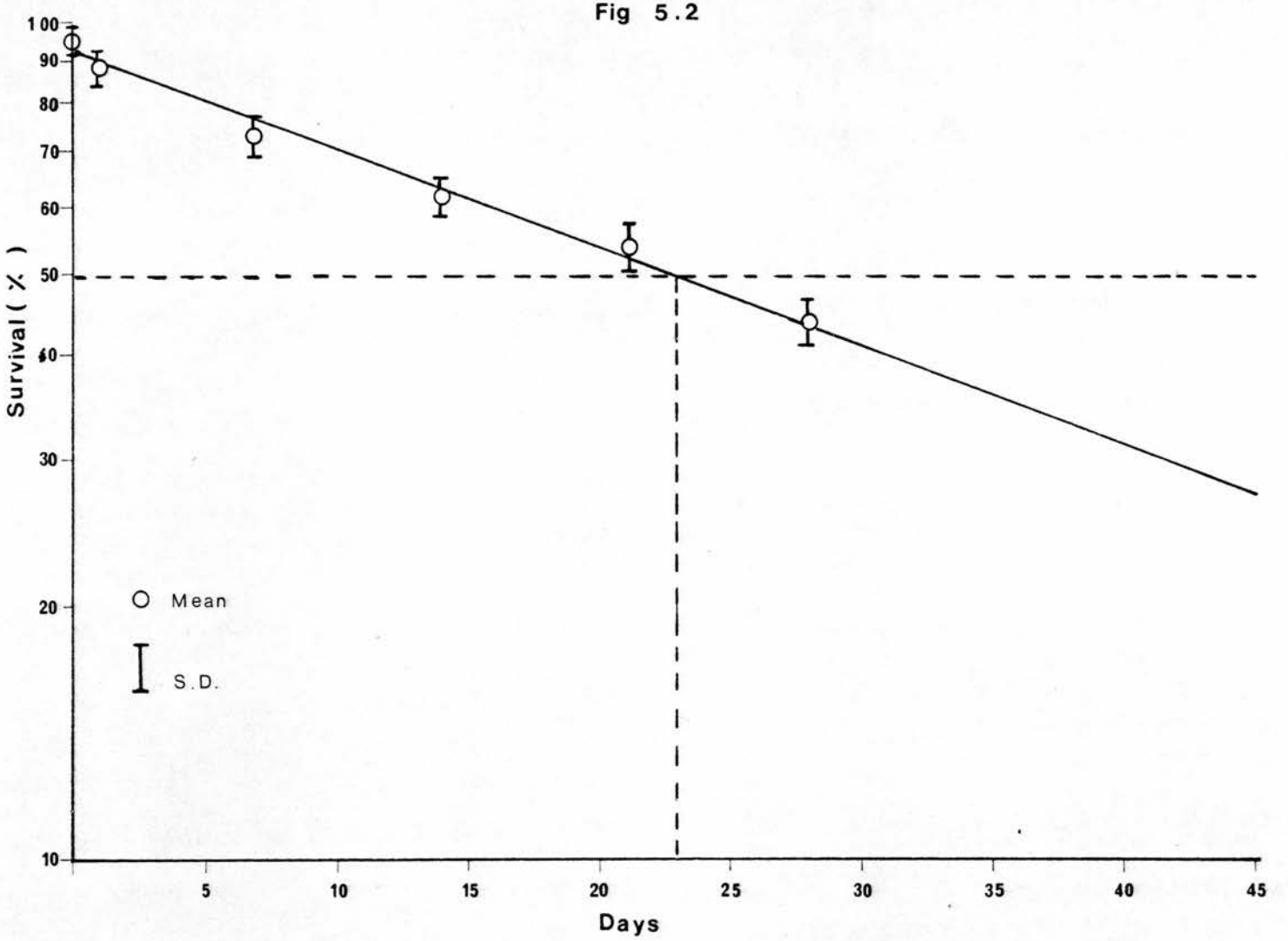
Summary for post-transfusion survival of frozen red cells processed by the IBM and transfused after 5 days

	Leucocyte count x 10^9/l			Platelet count x 10^9/l			Plasma haemoglobin				Post-transfusion survival(%) at					
	Pre tx	30min post	24-hr post	Pre tx	30min post	24-hr post	Pre tx	Trans-fused blood g/unit	Expec-ted g/l	obser-ved at 30min g/l	30 min	24-hr	(1) wk.	(2) wks	(3) wks	(4) wks
1. G.S.	6.4	10.8	N.D.	N.D.	N.D.	N.D.	0.107	0.82	0.36	0.46	84	83	N.D.	N.D.	N.D.	N.D.
2. J.D.C.	8.0	19.4	"	"	"	"	0.035	0.60	0.27	0.32	102	92	"	"	"	"
3. K.A.A.	8.2	8.4	"	"	"	"	0.039	0.35	0.16	0.28	93	87	"	"	"	"
4. A.E.R.	4.9	11.4	"	"	"	"	0.059	0.58	0.28	0.19	97	90	"	"	"	"
5. D.S.P.	N.D.	N.D.	"	"	"	"	N.D.	N.D.	N.D.	N.D.	85	74	"	"	"	"
6. K.A.	6.8 N% 51 L% 47 M% 1 E% 1	4.6 40 57 2 1	5.9 N.D. " " "	154	124	195	0.06	0.59	0.25	0.25	95	87	71	62	52	43
7. D.P.	5.4 N% 62 L% 29 M% 6 E% 3	11.8 78 17 3 2	6.2 67 27 6 0	139	160	240	0.02	1.19	0.42	0.37	92	94	70	61	49	42

TABLE 5.3 (cont'd.)

	Pre tx	30min post	24-hr post	Pre tx	after 30min	after 24-hr	Pre tx g/l	trans-fused blood g/unit	Expec-ted g/l	obser-ved after 30min	30 min	24-hr	(1) wk.	(2) wks	(3) wks	(4) wks
8. C.D.	6.6	15.5	8.8	200	195	200	0.022	1.24	0.35	0.23	97	85	75	61	56	44
	N 71	81	69													
	L 27	18	26													
	M 2	1	4													
	E 0	0	1													
9. A.R.	4.0	9.6	4.5	210	215	N.D.	0.002	1.10	0.39	0.30	98	86	71	65	56	43
	N 47	77	N.D.													
	L 49	18	"													
	M 2	4	"													
	E 2	1	"													
10. J.B.	8.3	14	7.9	180	210	175	0.05	0.27	0.15	0.11	99	91	77	66	55	47
	N 71	75	67													
	L 26	21	25													
	M 3	4	7													
	E 0	0	1													
Mean	6.5	11.7	6.7	177	181	203	0.04	0.75	0.29	0.28	94	87	73	63	54	44
S.D.	1.52	4.3	1.7	30	38	27	0.03	0.36	0.10	0.10	5.9	5.7	3.0	2.4	3.0	1.9

Fig 5.2


100
90
80
70
60
50
40
30
20
10
Survival (%)
Mean
S.D.
5
10
15
20
25
30
35
40
45
Days

OBSERVATION ON THE RECIPIENT LEUCOCYTE AND PLATELET COUNTS AFTER THE TRANSFUSION OF AUTOLOGOUS FROZEN BLOOD

During the early experiments of the autologous in vivo survival of frozen red cells we noticed a significant change in the leucocyte count of the recipient as compared to the pretransfusion level. This observation was interesting since we know that the frozen blood is deficient in leucocytes. We extended our observation to the differential count of the recipient as well as the level of the platelets. The result of these observations are shown in Tables 6.4 and 6.5. The mean total leucocyte count prior to transfusion was $6.45 \pm 1.21 \times 10^9/1$ (n=15). The mean total leucocyte count of the recipient 30 minutes after transfusion of one unit of autologous frozen blood was $13.0 \pm 4.7 \times 10^9/1$. Twenty-four hours post-transfusion the level of the leucocyte count decreased again and the mean count became $6.73 \pm 1.72 \times 10^9/1$. Thus, there was a significant increase (t-test = 5.5405 $p < 0.0005$ $n_1 = 12$, $n_2 = 15$, respectively) in the level of the total leucocyte count in the recipient 30 minutes after transfusion of one unit of autologous frozen blood when compared to those prior to transfusion (Table 5.4). Table 5.4 shows also that this increase was the result of increase in the neutrophil leucocytes. As regards the platelet count the mean count in 10 individuals prior to transfusion was $173.0 \pm 23.9 \times 10^9/1$. After 30 minutes of transfusion of one unit of autologous blood the count was not statistically significant from the pre-transfusion level. However, twenty-four hours post-transfusion the platelet count has increased to levels higher than those of the pre-transfusion with a mean of $202 \pm 32.4 \times 10^9/1$. This increase was statistically significant (t-test = 11.5169 $p < 0.0005$ n_1 (pre-transfusion) = 10, n_2 (24 hour post-transfusion) = 9.

TABLE 5.4

Effect of autologous transfusion of frozen blood on the leucocyte count of the recipient

	LEUCOCYTE COUNT X $10^9/l$											
	Total	Pre-transfusion Differential (%)				Total	30 min post-transfusion Differential (%)				Total	24-hr. post-transfusion Differential (%)			
		N	L	M	E		N	L	M	E		N	L	M	E
J.C.	7.0		N. D.			19.1		N. D.			N.D.		N. D.		
D.P.	6.9		N. D.			12.9		N. D.			N.D.		N. D.		
C.D.	6.1	58	35	6	1	10.5	76	17	6	1	7.7	66	28	4	2
J.C.	6.4	64	31	3	2	16.7	86	14	0	0	5.6	76	16	5	3
M.C.	8.0	77	15	8	0	24.2	77	18	3	2	9.1	57	38	5	0
A.R.	5.3	ND	ND	ND	ND	10.3	ND	ND	ND	ND	4.5	73	24	3	0
D.B.	6.5	61	37	1	1	12	74	22	3	1	6.5	55	40	4	1
G.S.	6.4	ND	ND	ND	ND	10.8	ND	ND	ND	ND	N.D.	ND	ND	ND	ND
K.A.	8.2	"	"	"	"	8.4	"	"	"	"	9.0	"	"	"	"
A.R.	4.9	"	"	"	"	11.4	"	"	"	"	5.0	"	"	"	"
D.P.	5.4	62	29	6	3	11.8	78	17	3	2	6.2	67	27	6	0
K.A.	6.8	51	47	1	1	4.6	40	57	2	1	5.9	ND	ND	ND	ND

TABLE 5.4 (cont'd.)

	LEUCOCYTE COUNT X $10^9/l$													
	Total	Pre-transfusion Differential (%)				Total	30 min post-transfusion Differential (%)				Total	24-hr. post-transfusion Differential (%)			
		N	L	M	E		N	L	M	E		N	L	M	E
C.D.	6.6	71	27	2	0	15.5	69	28	3	0	8.8	69	26	4	1
A.R.	4.0	47	49	2	1	9.6	77	18	4	1	4.5	ND	ND	ND	ND
J.B.	8.3	71	26	3	0	14.0	75	21	4	0	7.9	67	25	7	1
Mean	6.45	62	33	4	1	13.0	72	24	3	1	6.73	66	28	5	1
S.D.	1.21	9.7	10.6	2.5	1.07	4.7	12.9	13.2	1.6	0.78	1.72	7.2	7.7	1.3	1.07
n	15	9	9	9	8	15	9	9	9	9	12	8	8	8	8

TABLE 5.5

Effect of autologous transfusion of frozen blood on the platelet count of the recipient

	Platelet count x $10^9/l$		
	Pre-tx	30min-post Tx	24-hr post Tx
C.D.	155	124	155
J.C.	200	185	225
M.C.	170	165	255
A.R.	154	150	190
D.B.	165	160	180
J.B.	180	210	175
C.D.	200	195	200
A.R.	210	215	N.D.
K.A.	154	124	195
D.P.	139	160	240
Mean	173.0	169	202
S.D.	23.9	32.2	32.4
n.	10	10	9

Effect of transfusion of frozen blood on the recipient plasma free haemoglobin:

The mean plasma haemoglobin level prior to transfusion was 0.05 $\pm$ 0.05 g/l n = 14.

The total free haemoglobin in the supernatant per unit of blood transfused ranged from 0.263g to 2.387g. The total volume of blood transfused to each individual together with the expected plasma haemoglobin after transfusion are shown in Table 5.6. The change in plasma haemoglobin after the infusion of the frozen blood is also shown in the Table. In three out of 14 cases there was a slight haemoglobinaemia in excess of the expected levels. Haemoglobinuria was not observed in any of the recipients tested in this Table.

TABLE 5.6

Effect of transfusion of frozen blood on the plasma haemoglobin of the recipients

Name of the recipient	Plasma Hb Pre-transf. g/l	volume of transfused blood(ml)	supernatant Hb in the transfused blood(g/unit)	Expected plasma Hb of the recipient g/l	observed plasma Hb of the recipient g/l	Duration of storage post-thaw (days)
1. C.D.	0.033	183	2.159	0.70	0.236	0
2. J.D.C.(1)	0.031	204	2.387	0.744	0.272	0
3. M.C.	0.221	150	0.854	0.385	0.371	0
4. A.E.R.(1)	0.035	167	0.263	0.128	0.118	0
5. D.B.	0.019	259	0.988	0.366	0.301	0
6. G.S.	0.107	187	0.826	0.36	0.46	5
7. J.D.C.(2)	0.035	227	0.602	0.278	0.32	5
8. K.A.(1)	0.039	245	0.351	0.161	0.279	5
9. A.E.R.(2)	0.059	210	0.575	0.282	0.188	5
10. D.S.P.	0.02	400	1.192	0.417	0.37	5
11. K.A.A.(2)	0.06	326	0.585	0.254	0.250	5
12. J.B.	0.05	282	0.268	0.152	0.11	5
13. C.D.	0.022	408	1.243	0.354	0.23	5
14. A.E.R.	0.002	304	1.10	0.385	0.30	5
Mean	0.05	254	0.96	0.35	0.27	
S.D.	0.05	82	0.65	0.19	0.10	
n.	14	14	14	14	14	

LEUCOCYTE CONTENT OF THE FROZEN BLOOD

Since, in the last few years, preparation of leucocyte poor blood has received much attention, we have investigated our frozen blood for its leucocyte content. First we studied the leucocyte content of the blood before and after freezing, thawing and processing. Leucocyte counting was performed electronically by the Coulter counter and expressed as millions per gram haemoglobin.

The leucocyte content of the blood prior to freezing was 50.16 (million/g Hb) $\pm$ 28.91 in one series n = 17 (Table 3.16) and 49.09 (million/g Hb) $\pm$ 20.19 in another series n = 88 (Table 4.26). The leucocyte content of the blood after glycerolization, freezing and washing decreased to 2.85 $\pm$ 2.15 million/g Hb n = 37 for blood processed by the manual method (Table 3.12) and to 2.5 $\pm$ 1.2 million/g Hb n = 34 for blood processed by the IBM 2991 Automatic cell processor in one of the series (Table 3.13) and to 3.17 $\pm$ 2.12 million/g Hb n = 15 in another series (Table 3.16). This represents 5.7%, 5% and 6.3% of the leucocyte input before freezing respectively. The amount of leucocytes left in blood processed by the IBM 2991 was not significantly different from that left after manual washing ($t = 0.799$ $P > 0.1$ $n_1 = 34$ and $n_2 = 37$, respectively). In a third series processed by the IBM 2991 the residual leucocyte content of the resuspended blood after freezing, thawing and washing was 5.97 $\pm$ 4.32 million/g Hb n = 95 (Table 4.26). This represents 12% of the original leucocyte content prior to glycerolization and freezing. In comparing this with the previous series the only variable seemed to be the method of sampling. In the first two series, the whole units were sacrificed so that

sampling was direct from the processing bag after resuspension and thorough mixing, while in the latter case the samples were obtained from the line which contained most of the buffy coat. Stained smears prepared directly from processed blood and from leucocytes after concentration with dextran revealed the presence of mononuclear cells only, however the majority of these cells were distorted.

Recovery assay with labelled cells:-

Becuase of the fact that the mechanism by which leucocytes are removed from the blood during the various processes was not clear and because we believed that some of the leucocyte debris might still be present in the final product, we carried out what we call a recovery assay with labelled cells. A total of 31 units of blood were frozen after the addition of either lymphocytes, granulocytes or platelets, the cell membranes of which were labelled with radioactive ^{125}I - NaI. Thirteen units were used for the study of lymphocyte derived material, eight units for granulocytes and ten units for platelet studies. It was hoped that such a study might correlate with the previous method of assaying the leucocyte recovery and help us to get a better idea of the amount of cellular debris left in the blood after processing. Also we made use of this experiment to study the effect of passing the processed blood through the following filter systems:-

1) BR-10 Blood Administration Set (Baxter).
2) Ultipor - blood transfusion filter (Pall corporation)
3) Swank Transfusion Filter

Table 5.1 shows the results obtained with labelled lymphocytes. In the first group (Table 6.1a) of this series the blood was processed

in the IBM 2991 Automatic Cell Processor without removal of the buffy coat. It was found that about one third of the input labelled material was washed out in the waste, while about 40% of this material still remained in the final product. The rest of the labelled material was assumed to be in the processing bag. This latter material was usually found in the form of a gelatinous mass sticking to the wall of the processing bag. Sections of these gelatinous masses revealed the presence of few intact mononuclear cells, while most of the material was formed of white cell fragments.

There was a significant reduction (t-test = 3.0448 $p < 0.05$ $n = 6$) in the level of radioactive material in the processed blood after its passage through a BR-10 Blood Administration set when compared with that of the same blood before filtration. This suggests that some of the leucocyte debris are removed by this 170 μ filter.

Also there was a significant reduction (t-test = 2.27155 $P < 0.05$ $n = 6$) in the level of radioactivity of the processed blood after its passage through the blood transfusion filter when compared to that before filtration. However, there was no significant difference (t-test = 0.08463 $P > 0.475$ $n = 6$) between the radioactivity of the processed - Ultipor filtered blood and that of the same blood which passed through BR-10 Baxter Set.

The level of radioactivity in the processed blood was also significantly reduced (t-test = 2.8847 $P < 0.025$ $n = 6$) by passing the blood through a Swank-Transfusion Filter. The mean radioactivity remaining in the Swank filtered blood of the six units in this group was lower than that remaining after filtration through either BR-10 transfusion set or Ultipor-filter. This difference is statistically significant (t-test = 1.982 $p = 0.05$, $n = 6$).

In group II of this series (Table 6.1b) the buffy coat was removed at the end of the last wash step by manual overriding of the detector device of the machine, during which the upper layer of the packed red cells, containing most of the buffy coat, was removed. Under these conditions the amount of radioactivity remaining in the processed blood was decreased from about 40% in group I to 21% in group II. This difference was statistically significant (t - test = 3.964 $P < 0.025$ $n_1 = 6$, $n_2 = 4$ respectively). However this improvement was achieved by sacrificing more red cells (Table 6.1b). Table 6.1b shows that this decrease was the result of removal of more radioactive labelled material in the wash waste than that removed from blood in group I, the difference is statistically significant (t-test = 10.7155 $P < 0.0025$ $n = 4$). At the same time the amount of radioactive labelled material left in the processing bag in the form of gelatinous like masses was not significantly different from that in group I (t-test = 0.2756 $P > 0.4$ $n = 4$). Again when blood processed in this group was passed through a BR-10 Blood Administration set almost the same amount of radioactivity as in group I was removed. Neither the Ultipor nor the Swank filters were superior to the conventional BR-10 set in this group.

In the third group the buffy coat was removed, after each wash step. This resulted in washing out more radioactive material in the wash solution than that observed in the first or the second group. Thus there was a significant increase (t -test = 4.0258 $P < 0.01$ $n_1 = 3$, $n_2 = 4$ respectively) in the level of radioactivity detected in the wash waste in group III, when compared to that in group II. Also there was a significant reduction (t-test = 2.576 $P < 0.05$ $n_1 = 3$ $n_2 = 4$ respectively) in the level of remaining radioactivity in blood

processed under the condition of the third group when compared to that in blood of group II. Table 6.1c also shows that, when the buffy coat was removed after each wash step, there was no benefit in using any of the filters tested, as the amount of radioactivity remaining in the final product was not affected by any of them. Although removal of the radioactive labelled material in group III was maximal, under the condition of the experiment, the red cell loss was significantly higher (about 25%).

In order to prepare a pure granulocyte suspension for the study of the granulocyte derived material in the frozen blood, two units of fresh blood were filtered through a Leuko-Pak nylon filter. The effect of filtration through the Leuko-Pak filter, on the blood cell parameters is shown in Table 6.2. The absolute number and the per cent change in the white cell and platelet count due to filtration are shown in Tables 6.3a and 6.3b.

Table 6.4 shows that when previously frozen-thawed erythrocytes were washed in the IBM 2991 Automatic Cell Processor without removal of the buffy coat, approximately 25% of the input ^{125}I - labelled granulocyte material was recovered in the processed blood. The efficiency of the filters tested in removing the granulocyte derived material increased from the BR-10 Baxter set to the Ultipor-Pall filter to the Swank Filter. However, if the buffy coat was removed after each wash step, most of this material was removed with the wash solution and none of these filters were effective. Again in the latter case the red cell loss was as high as 25%.

As regards the platelet derived material present in the frozen blood, Table 6.5 shows that only about 2% of the platelet labelled

material remained in the final resuspended red cell product. This figure was not altered either by the technique of removal of the buffy coat or by the use of any of the filters tested.

Assessment of the viability of the remaining lymphocytes

Since it was evident from the previous experiments that, even after freezing, thawing and washing, some of the HL-A antigen containing cells and debris remain in the final product we attempted to assay the viability of those remaining cells and the antigen status of the blood as a whole. Whether or not the cells are viable may be critical, for in cases where the recipient is severely immunosuppressed, there is a risk of graft versus host disease. Again it is also important because viable cells may be more immunogenic than dead cells or cell fragments.

In this work viability of the remaining white cells was assayed by both the dye exclusion test and by the phytohaemaggutinin (PHA) stimulation test. However, both tests gave controversial results, for, while we observed that none of the cells examined were able to exclude the Trypan blue dye, the PHA test showed that, in nine out of 15 experiments, there was a weak response to PHA. When the latter was compared to the response of lymphocytes separated from the same blood before freezing and the results are expressed as a ratio, we found that this ratio varied from 4% to 30% with a mean of 13.44% $\pm$ 9 (Table 6.6).

In vitro assessment of the antigen status of the frozen blood:

The antigen status of the frozen blood was assayed in vitro utilizing the mixed lymphocyte reaction technique. In this method

lymphocytes separated from the frozen blood were incubated with viable, antigenically different, lymphocytes in a one way mixed lymphocyte culture. Antigenicity was measured in terms of the activity of tritiated - thymidine incorporated by one million responding cells. A positive control for this test was set up by producing a similar reaction between lymphocytes separated from the same blood prior to freezing and the same responding cells. In some experiments (1,2,3,4 and 5) (Table 6.7) the responding cells used were obtained from the frozen-stock of the tissue-typing laboratory. Also in these experiments, the fresh lymphocytes were tested for antigenicity on the day of donation while the lymphocytes from the frozen blood were tested approximately one year after. In either case the same batch of responding lymphocytes were used and their viability was assessed on each occasion (Table 6.7). A negative control was also included with each experiment consisting of the responding cells alone. A test was considered positive if the radioactive count observed was at least twice the negative control. In all the 15 experiments shown in Table 6.7 the mixed reaction with the fresh stimulating cells was strongly positive, while only one experiment, out of 15 utilizing frozen stimulating cells showed a positive reaction. However the latter might be an artifact as the baseline with this series of experiments was higher than the others, possibly due to contamination.

In vivo assessment of the antigen status of the frozen blood:

The in vivo tests of antigenicity were performed in rabbits. A preliminary experiment was performed in three groups of Dutch rabbits, one pair each. The first group received one ml of fresh blood, the

second one ml of frozen blood while the third was injected with one ml of frozen-Swank filtered blood. The Swank filter was selected because experiment with labelled cells showed that it is the best filter tested in removing the white cell debris. Injection of the rabbits was performed at weekly intervals and continued for 6 successive weeks. Fresh and frozen blood were from different sources. Serum samples were collected every week before the next injection. At the end of the experiment all the sera including the preimmunization sera were tested for the presence of anti-human lymphocytotoxic antibodies using random human lymphocytes as target cells. The result of this experiment is shown in Figure 6.1, where it can be seen that while rabbits which received fresh and frozen blood developed lymphocytotoxic antibodies after the first injection, it did not appear until the third dose in those receiving frozen Swank-filtered blood. Fig. 6.1 also shows that at any time of immunization rabbits receiving fresh blood produced a higher antibody titre than those receiving frozen or frozen-filtered blood and this holds true until the fourth week, after which all the rabbits showed a similar response.

Following these results the in vivo test for antigenicity was repeated with various modifications

1. Five rabbits were used in each group instead of two and immunization continued only for four weeks.
2. Blood used in each single week of immunization was obtained from the same donor for the three groups and all the donors used were of the same ABO group and Rh type.
3. Sera collected after immunization were adsorbed with pooled red cells from all the donors to remove the red cell antibodies.

4. The target cells used in the lymphocytoxicity test were pooled lymphocytes from the four donors.
5. Sera were diluted in double dilution and examined for lymphocytotoxic antibodies up to the end titre.

The response of each rabbit is shown in Tables 6.8a, 6.8b and 6.8c, while Figure (6.2) shows the mean response of each group. These results confirmed the findings of the preliminary experiments, except that rabbits receiving frozen blood did not respond by antibody formation after the first injection. Also in this experiment there was a marked difference between the antibody response of each group, the titre in the first group is $>$ the second $>$ the third.

TABLE 6.1a

Lymphocyte derived material assay with ^{125}I labelled lymphocytes

Group I. Lymphocyte derived material - buffy coat intact (Mean red cell recovery 94.8%)

Unit No.	% ^{125}I count remaining in					
	Wash waste	processed blood	Processed blood after passing through			processing bag
			giving set	Pall filter	Swank filter	
64597	N.D.	39.8	31.3	25.4	14.7	N.D.
46123	N.D.	37.6	23.5	23.5	23.5	N.D.
46132	39.9	49.0	38	40.8	17.5	11.1
46323	30.3	41.7	38.9	38.9	37.3	28.0
46336	33.6	41.7	34.6	34.6	33.8	24.7
11716	33.8	27.7	29.2	33	26.2	38.5
Mean	34.4	39.5	32.5	32.7	25.5	25.5
S.D.	4.0	6.97	5.81	7.01	8.87	11.3

TABLE 6.1b

Group II. Lymphocyte derived material - buffy coat removed at the last wash step (Mean red cell recovery 90%)

Unit No.	% ^{125}I count remaining in					
	Wash waste	processed blood	Processed blood after passing through			processing bag
			giving set	Pall filter	Swank filter	
64295	55	18.5	16.5	14.8	6	26.5
10467	52.3	30.5	18.2	14.6	14.2	17.2
10456	49.2	23.2	13.6	14.5	20.5	27.6
64289	50	12.9	11.8	12.8	14.8	37.1
Mean	51.6	21.3	15	14.1	13.8	27.1
S.D.	2.61	7.45	2.87	0.93	5.97	8.14

TABLE 6.1c

Group III. Lymphocyte derived material - buffy coat removed after every wash step (Mean red-cell recovery 75%)

Unit No.	% ^{125}I count remaining in					
	Wash waste	processed blood	Processed blood after passing through			processing bag
			giving set	Pall filter	Swank filter	
46254	79.5	11.6	14	10.7	9.2	9.9
28254	64.4	9.1	3.0	3.0	2.6	26.5
28262	91.0	8.5	6.2	6.0	5.7	0.5
Mean	78.3	9.73	7.73	6.57	5.83	12.3
S.D.	13.34	1.64	5.66	3.88	3.3	13.1

TABLE 6.2

Effect of filtration through Leuko-Pak filter on the blood cell parameters of fresh blood collected on heparin anticoagulant

Unit No.	W.B.C. $\times 10^9/l$	Platelets $\times 10^9/l$	R.B.C. $\times 10^{12}/l$	haemoglobin g/dl	Hct	M.C.V. (fl)	M.C.H. pg	MCHC g/dl
15009	Pre-filtration 5	235	5.42	14.7	0.452	84	27	32.5
	Post-filtration 1.9	66	5.38	14.6	0.447	84	27	32.7
15010	Pre-filtration 4.6	199	4.95	15.1	0.44	90	30.4	34.3
	Post-filtration 1.9	102	4.56	14.2	0.405	89	31	35.2

TABLE 6.3a

Efficiency of Leuko-Pak filter in removing leucocytes from fresh blood

Unit No. 15009

	Pre-filtration	Post filtration	% removal	% all granlocyte removal
Total W.B.C.($x10^9/l$)	5.0	1.9	62	
Neutro ($x10^9/l$)	1.950	0.342	82.5	85.2
Lympho ($x10^9/l$)	2.150	1.501	30.2	
Mono ($x10^9/l$)	0.550	0.057	89.7	
Eoso($x10^9/l$)	0.350	0	100	
platelets($x10^9/l$)	235	66	72	

TABLE 6.3b

Unit No. 15010

	Pre-filtration	Post filtration	% removal	% all granulocyte removal
Total W.B.C.($x10^9/l$)	4.6	1.9	59	
Neutro ($x10^9/l$)	1.840	0.418	77.3	
Lympho ($x10^9/l$)	1.932	1.425	26.3	78.9
Mono ($x10^9/l$)	0.690	0.057	91.8	
Eoso ($x10^9/l$)	0.138		100	
platelets ($x10^9/l$)	199	102	48.8	

TABLE 6.4

Granulocyte derived material assay with ^{125}I - labelled granulocytes

Unit No.	% ^{125}I count remaining in					
	Wash waste	Process-ed blood	Processed blood after passing through			Processing bag
			Giving set	Pall filter	Swank filter	
Group I: Buffy coat intact (Mean red cell recovery 94.8%)						
66489	60.6	22.4	17.5	13.4	9.7	17
66524	73.3	22.7	20.7	17.3	12.5	4.0
66522	53.3	22.9	22.5	20.5	13.5	23.8
51807	34.0	35.7	25.9	15.0	9.8	30.3
Mean	55.3	25.83	21.65	16.55	11.38	18.78
S.D.	16.43	6.59	3.51	3.0	1.92	11.25
Group II: Buffy coat removed after each wash step (Mean red cell recovery 74%)						
51550	71	8.9	9.5	7.5	11	20.1
14021	72.6	10.5	9.0	9.7	7.0	16.9
14086	52	10.5	10.0	9.5	8	37.5
51740	75	8.8	9.4	10.0	8.9	16.2
Mean	67.65	9.68	9.48	9.18	8.73	22.68
S.D.	10.56	0.95	0.41	1.14	1.7	10.0

TABLE 6.5

Platelet derived material assay with ^{125}I - labelled platelets

Unit No.	% ^{125}I count remaining in: Wash waste	processed blood	Processed blood after passing through: giving set	Pall filter	Swank filter	Processing bag
Group I: Buffy coat intact (Mean red cell recovery 94.8%)						
61106	N.D.	2.7	2.1	1.9	1.6	N.D.
61108	95	2.9	2.6	2.4	2.5	2.1
61209	N.D.	2.6	2.0	1.7	1.7	N.D.
61113	N.D.	2.7	2.2	2.2	1.6	N.D.
25954	96.6	1.9	1.5	1.4	1.4	1.5
Mean	N.D.	2.56	2.08	1.92	1.76	N.D.
S.D.		0.38	0.4	0.4	0.43	
Group II: Buffy coat removed after each wash step: Mean red cell recovery (76.22 ± 13.16%)						
61107	92	2.5	2.6	2.8	2.6	5.5
61109	100	1.6	1.7	1.5	1.7	0
61123	96	2.0	1.9	1.9	1.7	2
61180	101	2.0	2.1	2.0	2.3	0
25956	98.5	1.6	1.6	1.7	1.7	0
Mean	97.5	1.94	1.98	1.98	2.0	
S.D.	3.61	0.37	0.4	0.5	0.42	

TABLE 6.6

Comparison of the viability of lymphocytes separated from blood before and after freezing as measured by its response to PHA and expressed as the activity of H^3 CPM/Million cells

Unit No.	duration of storage frozen (days)	Response to PHA of test lymphocytes separated from: Fresh blood	Frozen blood	Ratio of response to PHA $\frac{\text{frozen}}{\text{fresh}}$ (per cent)
Non responders				
1. 13186	320	352,240	220	
2. 14590	398	241,038	< control	
3. 0460	323	91,543	2030	
4. 86	1	199,308	248	
5. 97	1	185,940	308	
6. 01452	1	169,340	528	
Mean		206,568	637	
S.D.		86,606	693	
Responders				
7. 01458	1	175,949	32,676	19
8. 02333	1	146,024	15,204	10
9. 02335	1	241,688	10,388	4
10. 01728	1	180,159	53,659	30
11. 01729	1	106,616	8,562	8
12. 01349	1	170,228	21,648	13
13. 01350	1	220,216	11,848	5
14. 01224	353	215,064	50,836	24
15. 13677	413	155,486	12,788	8
Mean		179,047	24,178	13.44
S.D.		41,678	17,516	9

N.B. The response in this table is expressed as the activity of tritiated-thymidine incorporated by 1 x 10^6 lymphocytes (CPM/million cells).

TABLE 6.7

Comparison of the antigenic status of fresh and frozen blood

Expressed as the activity of ^{3}H - thymidine incorporated per million lymphocytes when stimulated by antigenically different lymphocytes separated from blood, before and after freezing.

	Unit No.	duration of storage frozen (days)	* cells response to PHA (CPM/10^6cells)	** cells MLC CPM/10^6cells	*** cells MLC CPM/10^6 cells
1.	131686	320	88,324-100,340	65,318	< control
2.	13677	413	131,523-217,078	71,432	< control
3.	14590	398	117,978-170,095	108,367	447
4.	0460	323	81,241-272,993	59,435	2,384
5.	01224	353	130,848-254,216	68,048	< control
6.	01349	1	229,444	150,100	17,284 N.S.
7.	01350	1	229,444	151,236	5,416 N.S.
8.	01728	1	116,927	133,958	95,824 +VE
9.	01729	1	116,927	103,343	17,756 N.S.
10.	02333	1	360,224	130,860	< control
11.	02335	1	360,224	56,560	2,620 N.S.
12.	86	1	35,348	57,640	< control
13.	97	1	35,348	56,416	< control
14.	01452	1	175,762	111,246	< control
15.	01458	1	175,762	81,854	5,932 N.S.

* Viability of the responding cells assayed by its response to PHA and expressed as the activity of H^3 - thymidine incorporated by 1 x 10^6 lymphocytes.

** Antigenic status of lymphocytes separated from fresh blood before freezing and assayed by its ability to stimulate antigenically different cells in one way mixed lymphocyte culture and expressed as the activity of ^{3}H - thymidine incorporated by 1 x 10^6 responding cells.

*** Antigenic status of lymphocytes separated from the blood after freezing, thawing and processing and assayed by its ability to stimulate the same cells as in (**) and expressed in a similar way.

N.B. The stimulating cells in (*) and (**) belong to the same donor and were separated from the same donation.

Fig 6.1

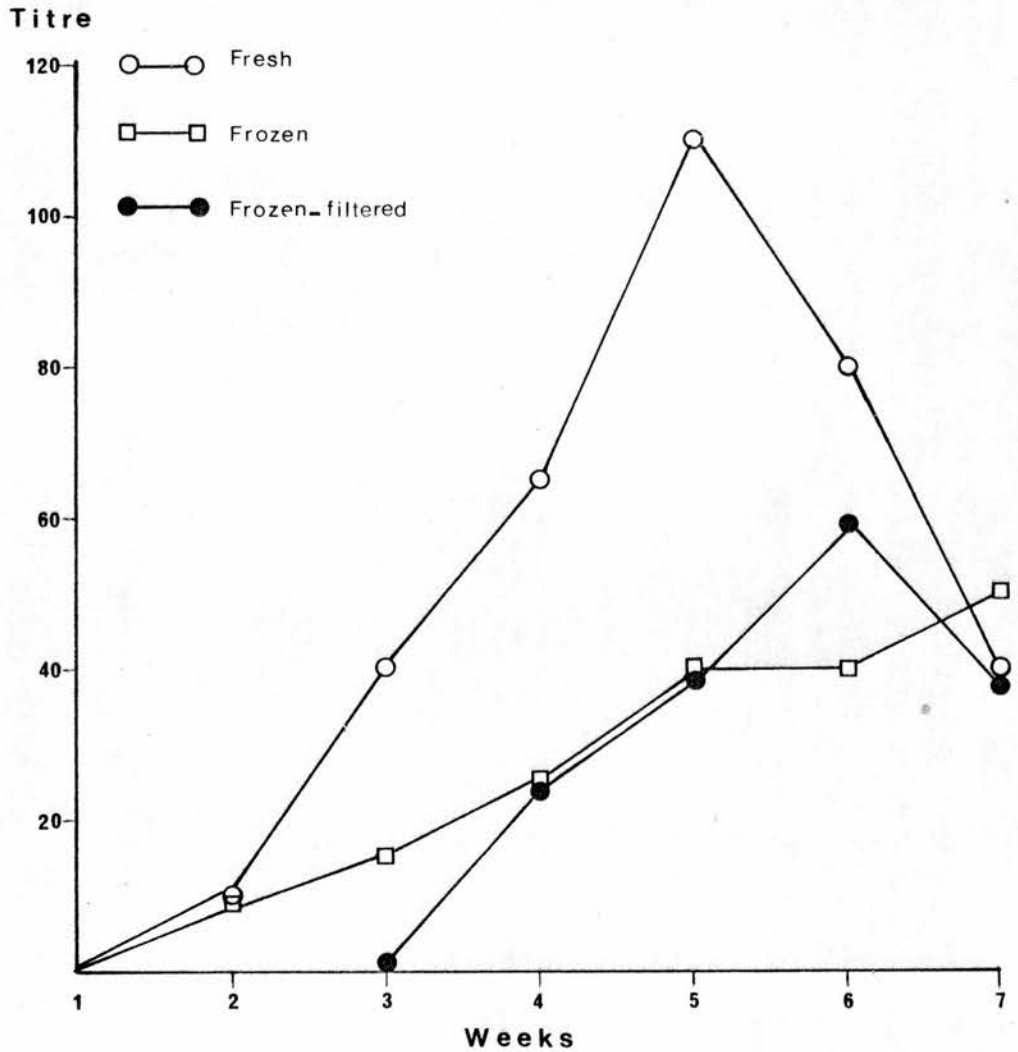

Titre
120
100
80
60
40
20
Fresh
Frozen
Frozen_filtered
1
2
3
4
5
6
7
Weeks

Fig 6.2

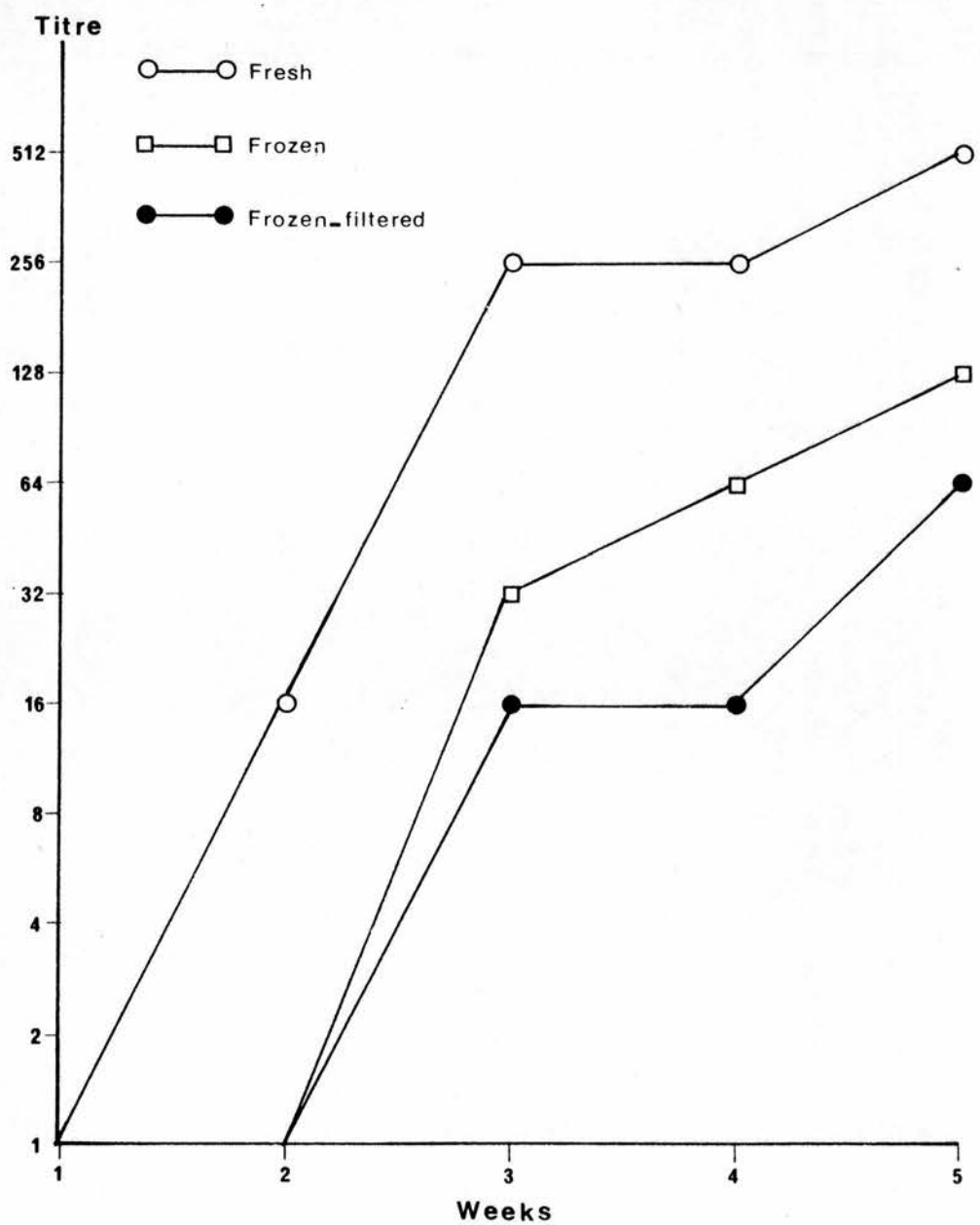

Titre
Fresh
Frozen
Frozen_filtered
512
256
128
64
32
16
8
4
2
1
1
2
3
4
5
Weeks

TABLE 6.8a

Titres of lymphocyte cyto-toxins after adsorption with pooled red cells

Fresh Blood

Group I	Rabbit No.1 titre	No. 2 titre	No. 3 titre	No. 4 titre	No.15 titre	Mean
Preimmunization	0	0	0	0	0	0
1st week	32	16	0	4	16	16
2nd week	512	128	32	512	64	256
3rd week	256	256	256	512	128	256
4th week	512	512	512	512	256	512

TABLE 6.8b

Frozen Blood

Group II	No. 10	No.11	No.12	No.13	No.14	Mean
Preimmunization	0	0	0	0	0	0
1st week	0	0	0	0	0	0
2nd week	16	64	64	16	32	32
3rd week	32	32	64	32	128	64
4th week	0	64	128	128	256	128

TABLE 6.8c

Frozen/Swank/Filtered Blood

Group III	No. 5	No. 6	No. 7	No. 8	No. 9	Mean
Preimmunization	0	0	0	0	0	0
1st week	0	0	32	0	0	0
2nd week	4	4	32	32	8	16
3rd week	8	16	16	16	32	16
4th week	32	64	2	8	128	64

N.B.

During preparation of this thesis, the anticoagulant in which the blood is collected during donation has been changed from ACD to CPD. We have, thus, examined the effect of this change on the characteristics of our final preparation. The results of this experiment is shown in table 7.1.

TABLE 7.1

Effect of post-thaw storage at 4°C in saline-ACD medium on the biochemical characteristics of blood collected in CPD before freezing

Unit No.	Pre-freeze			storage frozen	Immediately after washing						5-days post-wash				
	stor-at 4°C	ATP umol/ g Hb	pH		Recov (%)	pH	Spnt K^+ mmol/l	ATP umol/ g Hb	2,3DPG umol/ g Hb	Intra K^+ mmol/ 10^{12}RBC	pH	Spnt K^+ mmol/l	ATP umol/ g Hb	2,3DPG umol/ g Hb	Intra K^+ mmol/ 10^{12}RBC
67719	3	3.5	7.1	16	95.8	6.9	2.0	2.0	10	8.2	6.0	3.5	1.0	3.4	8.5
67737	3	3.6	7.3	16	95.2	7.0	1.5	2.3	6.9	8.6	6.2	2.8	1.2	1.8	8.5
67750	3	3.4	7.1	16	96	7.0	1.5	2.6	13.7	9.6	6.0	2.1	0.5	0	9.4
67752	3	2.1	7.3	16	94.6	6.9	2.0	1.9	12.9	8.1	6.1	1.9	1.0	0	8.0
56813	3	1.3	7.3	17	95.2	7.1	1.0	1.1	4.3	8.5	6.1	1.5	0.6	0.7	8.1
56814	3	1.8	7.2	17	97.2	7.0	1.0	1.3	8.5	8.5	6.1	1.7	0.8	2.4	8.7
56817	3	2.1	7.2	17	96.2	7.0	0.5	1.9	9.7	9.2	6.1	1.0	1.2	1.9	9.1
56825	3	1.9	7.3	16	80.4	7.0	0.5	1.1	12.2	8.7	6.0	1.3	0.8	2.5	8.8
56829	3	1.4	7.2	17	96.6	7.0	1.0	1.1	10.9	11.0	6.1	1.9	1.0	1.6	9.8
56832	3	1.8	7.4	17	95.3	7.1	2.5	1.3	12.4	9.3	6.1	2.9	1.2	4.6	8.7
56834	3	2.1	7.2	16	92.4	7.0	1.0	1.6	12.9	8.9	6.0	2.85	2.1	2.5	8.9
56839	3	2.3	7.3	17	92.4	7.0	2.5	1.7	10.3	8.5	6.2	2.5	1.2	1.8	8.5
Mean	3	2.3	7.2	16.5	94	7.0	1.42	1.7	10.4	8.93	6.1	2.16	1.1	1.9	8.75
S.D.	0	0.8	0.1	0.5	4.5	0.06	0.7	0.5	2.8	0.79	0.1	0.75	0.4	1.3	0.51

DISCUSSION

The attention of the Edinburgh and South East of Scotland Blood Transfusion Service was directed towards the freeze-preservation of human red blood cells to fulfil the need for a safe and efficient biologic product to supplement the present liquid stored blood programme. The main stimulus for establishing such a programme was the outbreak of Type B hepatitis in the renal dialysis and transplant units in which both patients and staff had died. This cause will remain valid as long as an absolutely certain method of detecting all potentially hazardous donations is not available.

It has frequently been reported that the incidence of Type B hepatitis is substantially reduced by transfusing previously frozen-thawed-washed erythrocytes (Haynes et al 1960, Haynes et al 1962, Murray et al 1962, Tullis et al 1970, Meryman 1972). In addition, clinical experience, has shown that frozen blood offers various advantages over the liquid stored blood. Thus, freeze preservation would enable stock piling of rare types of blood for transfusion to patients who are sensitized to blood-group antigens that are common in most donor populations. Frozen storage also makes possible an autotransfusion programme. Since the storage period in the frozen state is unlimited, freeze preservation would ensure the availability of blood of all groups and types at all times and reduce the wastage of outdating. Such a system, would thus, provide a means of equalizing supply and demand, and provide large stockpiles of frozen blood to cope with civilian or military catastrophes. Moreover, due to the fact that frozen blood is free of any plasma proteins, group "O" blood

can be regarded as a safer universal donation. Thus no minor cross matching will be required before its administration. Large stores of only group "O" Rh positive and Rh negative would make possible better blood bank management.

Other virtues claimed to be provided by frozen blood include reduction or elimination of the incidence of non-haemolytic transfusion reactions. This is claimed to be due to the removal of plasma protein (especially IgA), platelets and leukocytes. For the same reasons it has also been claimed that frozen blood is the most suitable preparation for patients in renal transplant and dialysis units as it may eliminate sensitization to HL.A antigens and hence avoid graft rejection.

Apart from those advantages, frozen-thawed-washed blood possesses additional advantages sufficient to justify its use. Thus, due to the washing procedures used to remove the additives, the final product has a very low content of free potassium, citrate, clotting factors and particulate matter. For these reasons the blood can be "tailored" with other needed components added, to suit individual requirements. Blood with low potassium levels is suitable for exchange transfusion in infants and for transfusion in patients with renal failure. Also blood which is devoid of coagulation factors may be suitable for administration to patients undergoing vascular surgery.

Since the demonstration by Smith, in 1950, that human red blood cells could be preserved in the frozen state and then recovered in high yields, a great deal of research and extensive effort has been invested in finding the optimal conditions for frozen storage. Various techniques of freezing have been described, which vary in

the nature of the cryoprotective, type of container, rate of freezing, storage temperature and post-thaw processing.

In recent years a considerable degree of uniformity has become obvious in the application of frozen blood banking. Thus, all methods of freezing human red blood cells in current use today employ glycerol, as an intracellular cryoprotective additive. Glycerol is used either in high concentration (40% w/v) combined with slow rate of freezing (1°C/minute) and storage at -80°C in a mechanical refrigerator, or in low concentrations (about 20% w/v) together with a fast freezing rate in liquid nitrogen and storage in the vapour phase of liquid nitrogen at -150°C. In either case glycerol must be removed from the red cells, by post-thaw washing, otherwise the preserved cells will haemolyse on transfusion. The high glycerol-slow freezing method was the first method described (Tullis et al 1958), and since that time it has been continuously improved until it has become a fully developed, practical technique used by most centres in the United States.

Nevertheless, this method has been repeatedly criticised on the grounds of difficulty in removing the high content of glycerol (Meryman 1968b, Pert and Schork 1969). In order to remove 40% w/v glycerol from the glycerolized-thawed red blood cells, it is necessary to use sophisticated equipment and large volumes of wash solutions (Valeri 1970) which makes the process expensive. The reasons that led the Americans to continue with this method of freeze-preservation appears to be related to their inability to use aluminium containers, which at the early stages of development was the only material suitable for liquid nitrogen temperatures, (-196°C). Aluminium was

not licenced for use in blood storage. By the time plastic bags had been developed, a large amount of money had already been invested in the development of mechanical refrigerators that operate at -80°C. Furthermore, the high cost of liquid nitrogen in the U.S.A. discouraged investigators from adopting the low-glycerol-rapid freezing technique.

In order to facilitate the post thaw washing procedure, the low glycerol-rapid freezing technique was recommended by Pert et al, 1963, Krijnen et al, 1964 and Rowe et al, 1968. Since its introduction, the method has gained wide acceptance in Europe for the difficulties encountered in its application in the U.S.A. were not valid in Europe. Thus, both the liquid nitrogen and labour costs are lower in Europe than in the U.S.A. Krijnen et al (1970) showed that aluminium cans could be used as blood containers for frozen storage without problems and the cost of the can has one tenth that of the plastic bag. This has made possible the establishment of frozen-blood banks in which low-glycerol-liquid nitrogen freezing in aluminium cans is coupled with manual batch washing. The latter enables the use of standard blood bank equipment and thus the heavy capital cost of automatic machines can be avoided.

Having investigated various choices or processes and equipment available worldwide, we chose the low glycerol-liquid nitrogen-aluminium can system, adaptated from the system used by Jenkins and Blagdon at Brentwood (1971). The can used in our system (69 mm x 160 mm) is slightly smaller than that used at Brentwood, (Jenkin and Blagdon 1971) and that used at Glasgow (Mitchell and Muir 1972). In this way we have been able to pack 204 cans, into a 250 litre Union Carbide refrigerator. This represents an advantage as regards

both space and running costs. A similar size of refrigerator is packed with 175 Printal cans at Brentwood (Blagdon personal communication) and 96 at Glasgow (Mitchell & Muir 1972). Finally the new can is of British manufacture and costs approximately half of the imported Printal can from Finland. Since the suggested can has different dimensions from those of the can in common use in Britain we have studied the optimization of variables. The critical parameter in frozen storage is the rate of freezing in the range $-4^{\circ}C$ to $-40^{\circ}C$ (Lovelock 1953a). This in turn is related to the can shape, size and finish and thus dictates the required glycerol concentration. For technical reasons it is convenient to keep the glycerol concentration as low as possible and this means a freezing rate as high as possible. It was originally believed that freezing of whole units of blood would require a can of large surface area and very small blood thickness (Strumia et al, 1958a). However, it is now realized that the limiting factor with such cans was not the thickness of blood, but the thickness of the insulating layer of vapour surrounding the can. In order to improve the rate of freezing, one has to increase the rate of heat transfer by reducing the thickness of the layer of vapour around the can (Luyet 1961). This was successfully achieved by application of a thin layer of thermally insulating materials to the outer surface of the metal container (Cowley et al 1961). Fortunately, it was found that the painted cans, as supplied, were already optimal. The observed mean freezing rate over the range $-10^{\circ}C$ to $-40^{\circ}C$ was 0.5 to $0.9^{\circ}C$ per second. Glycerol concentration was varied over the range 10% (W/V) to 35% (W/V) and found to be optimal at 21% (W/V (Pepper and Amer, 1972; unpublished work). In practice, this cannot be controlled to better than $\pm$ 1.5% W/V,

however, this is not important since the optimum curve is rather flat in this region (Pert et al 1965).

Starting a frozen blood programme according to the principles mentioned above was trouble free and has an important advantage over other blood freezing methods: a minimum requirement of special equipment (a liquid nitrogen refrigerator, aluminium cans for freezing and five-tailed plastic bags for washing). Post-thaw processing was performed manually using a conventional blood bank centrifuge and plasma expressor.

Having chosen the system of freeze-preservation we then investigated in detail other variables within the system which might affect the quality of the final product.

We have shown that the anticoagulant used in the collection of blood has no specific effect on either the in vitro red cell recovery or the biochemical characteristics of the final preparation immediately post-thaw. In this respect our results contradict those reported by Huntsman et al (1960) who found that EDTA had a better effect on the red cell recovery than that of ACD. However the results reported by these authors were obtained for blood frozen in droplet form without the use of a cryoprotective agent.

The finding that blood collected in EDTA or CPD behaves similarly to that collected in ACD after glycerolization, freezing, thawing and washing may be of some importance in cases where there is a shortage of blood, for if whole blood has been collected in EDTA and separated for the production of clotting factors II, VII, IX and X, then the red cells can be glycerolized, frozen and stored. On

deglycerolization, the EDTA is washed out of the erythrocyte suspension and thus the blood is suitable for transfusion.

Having found that the initial anticoagulant choice does not affect the immediate recovery of cells in vitro, we then went on to investigate the possible methods of washing. The choice lay between manual batch washing in five tailed bags as described by Blagdon (1972) or in one of the automatic machines. As it was financially impossible to purchase and operate all three commercially available machines, we studied their application in other blood banks and together with published figures decided that the IBM 2991 would be the most suitable machine for evaluation in the Edinburgh centre. We thus compared the manual batch wash with the IBM 2991.

Both manual and automatic batch washing have been evaluated and both methods have yielded excellent results.

Deglycerolization by manual batch washing required approximately one litre of wash solution and was quite efficient as demonstrated by the levels of the supernatant glycerol and sorbitol. The total amount of red cell loss during the preparation procedures was about 7.5%, of which one third was caused by the freeze-thaw process, whereas the rest was due to the washing steps. The least haemolysis was observed after the sorbitol wash where the red cells were maximally shrunken, whilst the highest haemolysis was noticed after the first saline wash step probably due to the rapid expansion of the shrunken cells in the isotonic medium.

The time required to process one unit manually was about 90 minutes, excluding thawing. However, four units of frozen blood could be processed simultaneously in the same centrifuge, by one operator,

in two hours.

Haematological examination of the final product revealed the presence of a few crenated red cells and a slight increase, (though still within normal limits)in the MCV, otherwise the cells appeared normal in both shape and haematological parameters. Microscopic examination of smears prepared from this blood also showed the presence of a few mononuclear cells, however, many of them were unidentifiable. No blood platelets have been identified in the examined blood films. The results of the biochemical analysis agreed with those reported by other workers and will be discussed later.

In vivo survival of the reconstituted manually processed cells was measured in five cases of auto-transfusion by the method described. The 24-hour post-transfusion survival ranged from 86% to 99% with a mean value of 93.4 ± 6.15.

Washing the frozen-thawed erythrocytes in the IBM 2991 Automatic Cell Processor utilized about one and a half litres of wash solutions. This is because of the larger capacity of the processing bag and the necessity of centrifuging it while completely full. The latter condition has also dictated a pre-wash dilution of the glycerolized blood with about 200 ml of wash I. However, this was found to have a beneficial effect on the final red cell recovery. The total red cell haemolysis is approximately 5% - half of which was due to freeze-thaw haemolysis while the rest was caused by the three wash steps.

We also observed that deglycerolization in the IBM 2991 was more efficient than that by manual batch washing, this was evidenced by the lower levels of residual glycerol and sorbitol in the supernatant.

The total processing time from thawing of the blood to the end of the wash cycle is approximately 20 minutes. Most of this time is spent in the automatic mode during which the technician is free to prepare for the next unit or to do other work.

The haematological and biochemical characteristics of the final product are not significantly different from those of blood processed by the manual technique. Again the in vivo survival was determined in five autologous transfusions by the ^{51}Cr and ^{125}I technique as described in this study. The results of the 24-hour post-transfusion survivals were not significantly different from those observed for red cells processed manually.

The manual method of processing offers the advantage of being cheaper to run as it does not require special equipment other than the processing bag. Any conventional blood bank centrifuge and plasma expressor would be sufficient for processing. On the other hand this method suffers from the following disadvantages:

1. It is time consuming - particularly if less than four units are processed at the same time.
2. It requires the full attention of the operator nearly all the time.
3. It requires many puncturing operations and thus we believe that blood washed by this method should be used within 12-24 hours of processing.

Automatic washing by the IBM 2991 Cell Processor has the following advantages over the manual method.

1. It produces a higher in vitro red cell recovery.
2. It washes more efficiently as judged by the sorbitol and glycerol concentration in the supernatant of the processed blood.

3. It saves both time and effort, as it does not require the operators attention except at the start and finish of process.

4. Blood processed by this method is potentially less susceptable to bacterial contamination, as the number of "open" manipulations are significantly reduced.

However, automatic processing by the IBM 2991 suffers also from the following disadvantages:

1. A higher capital cost of approximately £9000.

2. It is expensive to run - it adds on extra £4.0 per unit over the manual method for the disposable harness.

3. Only one unit of blood can be washed at a time and thus if four units of blood are to be processed, the machine would not provide much of a time saving when compared to the manual method.

Biochemical Characteristics of Liquid and Frozen Stored Blood

1. Intra and extracellular potassium:-

The results of measurements of intracellular potassium of fresh red blood cells by the method described in this study are slightly higher than those reported by Runck et al (1968). However this might be due to the different age of the blood at the time of measurement. While our value (9.91 m mol/10^{12} R.B.C.) represents the average intracellular potassium content in red cells after only one day storage at 4^{o}C, those of Runck et al (8.49 m mol/10^{12}R.B.C.) were obtained for red cells stored for up to six days. It is known that the intracellular potassium level is affected by the period of

storage at 4°C, the longer the period the lower the intracellular potassium (Runck et al 1968). For this reason, it can be argued that the reduction in intracellular potassium observed in this study, after glycerolization, freezing, thawing and washing, is due to prefreeze storage at 4°C rather than being due to the effect of these processes. This conclusion finds support from the observation that deglycerolization of frozen blood with ionic solutions in a continuous centrifugation system caused no significant change in their intracellular potassium (Runck et al 1968). In fact, in our experiments it is difficult to separate the effect of prefreeze storage from that of various processes involved in preparation of frozen blood in causing intracellular potassium depletion. However, it can be seen that all our measurements were performed on red cells that were stored at 4°C for approximately two days before freezing. Thus a difference of one day at 4°C between these cells and the control could not be responsible alone for the 20% reduction in the level of intracellular potassium observed. Also one has to consider the freezing rate used in each case and its effect on the ATP^{ase} and hence on the ATP and electrolyte content of the cell (Takehara and Rowe 1968). While Runck et al utilized slow freezing rate, our cells were frozen rapidly when the ATP^{ase} activity is high. In this respect the reduction in the intracellular potassium is consistent with the reduction of the 2,3 DPG and ATP observed in our experiments as a result of glycerolization, freezing, thawing and washing. Furthermore, when the fast freezing rate was used Valeri and Runck observed (1969b) a similar reduction in the intracellular potassium, although the authors were not able to specify its cause. From the practical point

of view, blood collected in any anticoagulant and stored at 4°C for two days, then glycerolized, frozen by our method, then thawed and deglycerolized will have approximately 80% of the intracellular potassium of fresh blood irrespective of the duration of storage in the frozen state. This is an important advantage of freeze preservation.

As regards the extracellular potassium we observed that storage of blood in ACD at 4°C resulted in potassium leakage from the red cells into the plasma so that after three weeks the extracellular potassium was as high as 16-28 m mol/l. These results were similar to those reported by Bunker (1966) and Whitcher (1964). Since high extracellular potassium concentrations are myocardiotoxic, physicians have always been concerned about the production of serious hyperkalaemia as a result of multiple transfusions of 'elderly' ACD stored blood. Freeze-preservation has the advantage of providing blood with a low extracellular potassium. We observed that the supernatant potassium of the frozen blood was usually between 0-2 m mol/l on the day of washing and rarely exceeded 5 m mol/l. Such a preparation would be beneficial for exchange transfusion in infants and for transfusing patients with renal failure and those who need massive blood replacement.

It is evident that the low extracellular potassium content of the frozen blood is the result of its thorough washing in order to remove the glycerol. However, during post-thaw storage, we observed a continuous increase in the supernatant potassium, and at the end of nine days storage in isotonic saline it may be as high as 14 m mol/l.

Supplementation of the isotonic saline with 70 ml of ACD substantially reduced the extracellular potassium so that after five days storage at 4°C it was only 6.5 m mol/l and reached 8.9 m mol/l after nine days. The reason for this reduction will be discussed later.

2. 2,3 Diphosphoglycerate

Much information has been published concerning the role of 2,3 DPG in the oxygen transport function in health and disease, however, the normal level of this compound in fresh blood is reported in only a few papers. Also the concentrations of the 2,3 DPG is expressed differently in different reports. Some investigators relate it to the red blood cells and the value is given as μ moles/ml R.B.C. or μ moles/10^{10} R.B.C. Others relate the compound to the haemoglobin and expressed their values as μ moles/mole Hb or μ moles/g Hb. This has resulted in some confusion and made the comparison between values from different sources difficult. However, in the recent literature, a uniformity in expressing the 2,3 DPG concentrations as μ moles/g Hb was noticed. Therefore, we have adopted the latter terminology throughout this study.

The level of the 2,3 DPG in previously frozen-washed erythrocytes is dependant on the initial amount, which in turn is determined by the age of the blood prior to freezing. Normal levels of the compound in fresh blood, as measured by the method described in this study, were 13.01 $\pm$ 1.8 μ mole/g Hb. These results agree with those reported by Fortier et al 1969, Valeri and Hirsch 1969, and Valeri and Zaroulis 1972). However, during storage at 4°C the 2,3 DPG is rapidly depleted from the red cells with a rate determined by the pH

of the anticoagulant used at collection, the lower the pH, the higher the rate. Thus we observed that during storage in ACD at 4°C, the level of 2,3 DPG fell to 70% after two days and 57% after three days and could not be detected after nine days. This would explain the wide variation in the concentrations of the 2,3 DPG observed when blood of different ages was used for freezing.

An additional fall in the levels of 2,3 DPG by about 20% was observed due to the processes of glycerolization, freezing, storage in the frozen state, thawing and washing. This reduction was observed with both the manual and automatic technique of processing and was not affected by the period of storage in the frozen state. This implies that if satisfactory levels of 2,3 DPG are to be conserved by freeze preservation, the blood should be frozen within the first three days of donation. Under these conditions the final red cell suspension would be better than ordinary 7-10 day old banked blood as far as 2,3 DPG is concerned.

3. Adenosine Triphosphate

The importance of ATP in the red cells has been discussed in the introductory section. Unlike 2,3 diphosphoglycerate, red cell ATP is present in lower concentration (approximately one fourth that of 2,3 DPG). For reasons of simplicity the value of this compound was also expressed as μ mole/g Hb. Initial values in fresh blood as measured in this study varied from 2.34 to 4.17 μ mole/g Hb which agrees with those reported by Fortier et al 1969, Valeri and Hirsch 1969, and Valeri and Zaroulis (1972). Again these levels are

decreased during storage at 4°C. However, contrary to 2,3 DPG, the lower the pH the slower the rate of ATP reduction. Thus we found that during storage in ACD at 4°C, the level of ATP was 90% of that in fresh blood after three days, 70% after nine days and 37% at the end of two weeks.

Similarly to 2,3 DPG, the level of ATP in the frozen blood is dependant on the age of the blood prior to freezing. Also an additional loss might be expected during processing. This was observed with both manual and automatic washings and sometimes was high as 34%. The latter values might be produced by the higher activity of the enzyme ATP-ase, which is stimulated by rapid rates of freezing and thawing (Takehara and Rowe 1968). However, by lowering the pH of the wash solutions or by immediate addition of ACD to the red cells after processing, this high loss was substantially reduced, this point will be fully discussed later in the post-thaw stability section.

4. Glycerol and Sorbitol Content in the frozen blood

Glycerol appears to be nontoxic when transfused to recipients, as it is an intermediate product of metabolism, and it has been shown that human subjects can tolerate the injection of 50 grams of glycerol contained in one litre of solution (Sloviter et al 1957). However, the transfusion of erythrocytes that contain a high concentration of glycerol renders them osmotically unbalanced and results in their haemolysis. It is for this reason that various methods of washing have been proposed. Since some glycerol is compatible with red cell survival it is not necessary to remove all the glycerol added. An

arbitrary limit of 1 g% was empirically introduced as the highest permissible level to be transfused with the red cells (Valeri et al 1969). The amount of the residual glycerol has often been thought to reflect the efficiency of the washing procedure. With the manual method of processing the residual glycerol in the supernatant varied from 0.48 to 1.26g/100 ml with a mean of 0.84 $\pm$ 0.26g/100 ml. Whereas the amount of glycerol remaining after washing the blood in the IBM 2991 ranged from 0.03 to 0.816g/100 ml supernatant, with a mean of 0.19 $\pm$ 0.22g/100 ml. This difference was statistically significant (Table 3.14) and thus establishes an advantage of the IBM 2991 over manual batch washing.

As regards the sorbitol, although it does not penetrate the cell membrane and hence it represents no problem as far as the red cell is concerned, because of its low molecular weight, it is osmotically active. Thus if present in sufficient concentration it may cause an increase in the recipient's plasma osmotic pressure with its side effects (Pert et al 1964). Therefore, the sorbitol added to the blood, with the glycerolizing solution or as the first wash, has to be removed or reduced to a minimum before transfusion. Here again, we found that the IBM 2991 was superior to the manual technique in removing the sorbitol from the blood. Thus, whilst red cells processed manually have a residual supernatant sorbitol ranging from 0.22 to 1.59g/100 ml, with a mean of 0.76g/100 ml those processed in the IBM 2991 have residual sorbitol concentrations ranging from 0.12 to 0.756g/100 ml and a mean of 0.29g/100 ml.

5. Aluminium content in the frozen blood

In 1970 Berlyne et al reported the incidence of hyperaluminaemia in three out of six patients with renal failure who were exposed to dialysate with relatively high aluminium content. Although the consequences of high serum aluminium was not known, the authors recommended avoiding administration of drugs containing the metal in cases of renal failure. In 1971, Waldron - Edward et al studies the effect of hyperaluminaemia on human metabolism. They reported that hyperaluminaemia has an adverse effect on activity of some of the physiological enzymes. This was evidenced by the decreased level of serum intestinal alkaline phosphatase, acid phosphatase and adenosine triphosphate among people with high serum aluminium. They also found a significant increase in the prothrombin time in these individuals.

For these reasons and because of the possibility of leaching out of aluminium into the blood, we decided to study the aluminium content in the final red cell preparation. Aluminium measurement was performed by Atomic Absorption Spectroscopy at the Laboratory Government Chemist. Levels of aluminium in normal blood as determined by this method varies from 7 to 11 μ mol/l (Berlyne et al 1970 and Waldron-Edward et al 1971). Earlier results showed some very high levels, however we have been asked to ignore them as the technique used was not very accurate. Later, some improvements were introduced to the method and since then the results have rarely exceeded 11 μ mol/l. It is evident that the levels of aluminium present in frozen blood are in the same range as those observed in vivo and thus there is no possible risk from using aluminium cans.

Post-Thaw Stability

After several years of comparative experience we have adopted the IBM 2991 Cell Washing Machine because it suits our ultimate method of issuing frozen blood in addition to its advantages in efficiency of washing and automatic operation. It is our experience that if patients are to receive frozen blood whenever necessary, the processing delay, especially at night, must be eliminated. Therefore we decided to operate a small bank of previously frozen-thawed-washed cells which had been "speculatively" thawed (six "O" positive and three "O" negative). As existing limitations of storage suggested that haemolysis was the major limiting factor in the shelf-life, we began an intensive study to optimise resuspension fluid and thus prolong the shelf-life considerably. However, such a biochemical improvement would be negated if the bacteriological status of the product was unsatisfactory. Therefore we had to show that both conditions could be satisfied simultaneously.

Despite all the advantages provided by the frozen blood it remains expensive and limited in application, unless considerable losses are accepted, because the shelf-life of the red cells once thawed and washed is restricted to less than 24 hours (Blagdon 1972). This restriction was based upon the instability of processed cells stored at $4^{\circ}C$, manifested by spontaneous haemolysis, and the potential risk of bacterial contamination occurring during open processing. The short shelf-life of frozen thawed blood is a serious disadvantage to its wide spread application.

Spontaneous haemolysis in physiological media is a common feature of all frozen-processed erythrocytes irrespective of the method used

for its preparation (Doebbler and Rinfret 1959) and is demonstrated by the high and rapidly increasing levels of haemoglobin and extracellular potassium in the supernatant. However, the rate of this haemolysis varies from one resuspension medium to another. Thus, the level of supernatant haemoglobin was found to be lower with ACD-plasma, as resuspension medium than that with 5% albumin (Tullis et al 1958 and Valeri 1965c).

However, neither the plasma nor the albumin would fulfil the requirements of an ideal resuspension medium to be used with the frozen blood. In the first place, the observed supernatant haemoglobin was high on the day of deglycerolization (up to 1750 mg per unit with the plasma and to 2365 mg/unit with albumin) and increased further on storage to 3150 mg and 6130 mg per unit respectively. Secondly, resuspension in plasma would deprive the frozen blood of many of its advantages namely the absence of transfusion hepatitis (Haynes et al 1960) and decreased incidence of urticarial and transfusion reactions which were due to the removal of immunoglobins and other plasma proteins. Finally the use of plasma for resuspension would certainly effect the protein fractionation programme and compromise the component therapy programme.

Using 5% albumin for resuspension of frozen-washed red cells, despite the fact that it avoids some of the disadvantages observed with plasma, can hardly be justified in terms of good blood bank management, due to its high cost.

Extracellular free haemoglobin has always been a matter of great concern as it was believed to represent one of the major difficulties

encountered with frozen blood transfusion. It is known that some of the frozen preserved cells might be irreversibly damaged so that on transfusion they are immediately removed from the circulation. If the removal of these cells is not accompanied by haemoglobinaemia, then the determining factor of acceptability, as far as the free haemoglobin is concerned, will be the level of the free haemoglobin in the supernatant fluid. The physiologically acceptable levels of free haemoglobin has not been accurately determined, it is believed to be a matter of individual variability related to a number of different factors. Of utmost importance, in this respect, is the level of the haemoglobin-binding protein (haptoglobin) in the recipient plasma. The normal level of this compound was found to be 1.068 $\pm$ 0.259 g/l for males and 0.827 $\pm$ 0.202 g/l for females (Mollison 1972). Following infusion, the free haemoglobin will be bound by the haptoglobin in the recipients circulation, until the latter is saturated (1.0g haemoglobin per one litre plasma). This haemoglobin-haptoglobin complex, due to its large molecular size, does not appear in urine but it is slowly removed by the reticuloendothelial system, predominantly by the liver. Free haemoglobin in excess of the haemoglobin-binding capacity will circulate as such with the blood until its level exceeds 25 mg/dl it starts to appear in urine (Mollison 1972). However, high levels of free haemoglobin in the supernatant are not uniformally toxic, for levels as high as 1.5 gram of haemoglobin and stromal protein per Kg body weight have been well tolerated on administration into rabbits (Zwilling 1958). It is probable that there is a great difference between the free haemoglobin and stroma produced as a result of incompatible transfusion and those

infused with the frozen transfused blood provided that the cells from which they are derived are immunologically compatible. However, although haemoglobinuria might be well tolerated by healthy individuals as well as patients with normal blood pressure and normal blood volume, it might, in seriously ill or shocked patients, precipitate acute renal failure (Valeri 1970).

Another hazard produced as a result of the instability of the frozen-thawed-washed erythrocytes on storage in physiological media at 4°C is the rapid increase in the free extracellular potassium in the supernatant fluid. This accumulated potassium is the result of leakage from intact cells and liberation from haemolyzed erythrocytes. A high level of extracellular potassium would deprive frozen-washed red cells of another advantage, which is its suitability for massive transfusion, and exchange transfusion in new-born infants. Furthermore, a substantial decrease in the intracellular potassium is corrolated with a decrease in the in vivo survival of the red cells (Valeri and Runck 1969a).

Prolongation of the shelf-life of the previously frozen-thawed-washed erythrocytes at 4°C would thus be an important achievement which would help in solving some of the difficulties encountered in this aspect of blood transfusion.

During preliminary studies with the IBM 2991 Automatic Cell Processor we realized that it offered the advantage that the end-product might be less susceptible to bacterial contamination as the number of "open" manipulations was significantly reduced. A planned study was, thus, undertaken to examine the incidence of bacterial

contamination in 100 units after post-thaw processing and storage for 10 days at 4°C in saline-ACD medium. The results of this study confirmed the earlier observations.

Units which showed contamination at zero day of processing came out as negative on colony count test and on late repeated culture as well as when cultured after 10 days of post-thaw storage. This might be interpreted as follows:

a) The early contamination was slight and happened to be picked up in the sample taken, and thus could not be detected by the colony count test. A negative repeated culture, in this case, would mean that the organism detected has died due to the hostile environment.

b) A second explanation is that the blood was sterile but contamination was introduced during the sampling process.

Out of the hundred units examined in this study only one unit showed bacterial growth after ten days of post-thaw storage at +4°C. Although the incidence of bacterial contamination was confirmed by repeated cultures, the colony count test was negative which mean that the number of the organism present was low, and inconsistent with contamination having occurred ten days previously. An incidence of $\leq$ 1% after 10 days of post-thaw storage at +4°C could be considered as a low rate especially when compared with that of the fresh blood which is variously reported to be from 1 to 3% (Braude et al 1952, Walter et al 1957 and Parikh and Kulkarni 1967).

It is very gratifying that the bacterial examination of unused, thawed-washed blood which has been prepared for routine clinical use and stored for 6-10 days has shown no contamination in over 500 units.

We suspect that many if not all of the observed contaminant organisms in the first trial of 100 units were introduced by the sampling technique and therefore are artifacts.

Approximately one thousand units of frozen-thawed-washed blood have been transfused after storage for one to five days without any incidence of bacterial reaction.

In vitro instability of the frozen-thawed-washed erythrocytes, when stored in physiological media at $4^{o}C$, probably results from the effect of glycerolization, freezing, thawing and deglycerolization which bring about a certain degree of damage to the red cells. When the damage produced is severe, the cellular integrity is lost and the cells are lysed during processing. A less intensive assault will result in irreversible damage, but the cells will remain intact, and on transfusion they are immediately removed from the circulation. Still other cells might be reversibly affected by the processes of glycerolization, freezing and washing as evidenced by the substantial increase in their intracellular sodium and decrease in potassium ion concentrations, however upon transfusion they are rapidly restored to normal (Crawford and Mollison 1955, Valeri and Hirsch 1969) and show a normal survival (Valeri 1971). The fact that the resuspension media are unable to reverse the damage produced in the frozen-washed cells would imply that these media either lack a substance which is otherwise found in the circulating plasma or that a substance essential for maintaining the viability of the cells, is rapidly depleted on processing and post-thaw storage. The intravascular environment probably provide suitable circumstances for the regeneration of this vital substance or substances.

In normal red cells, cation movement across the cell membrane is mainly an active process that takes place against a concentration gradient (pump) and in which sodium ions are driven outside the cell in exchange for potassium. This process is energy-dependant, which is provided by ATP (Hoffman 1966). The role of this sodium-potassium pump may be to compensate for the changes that result from the continuous passive leakage of these two ions into and out of the cell. Since the cells that suffered from reversible damage as a result of freezing, thawing and washing, show an increase in their intracellular sodium and a reduction in the potassium concentrations then this would suggest a dysfunction of the ATP - dependent cation transport. The latter probably occurs due to a reduction in the intracellular ATP content. Such a reduction has been previously reported to occur due to the freeze-thaw processing (Hurn 1968) and is confirmed from our results (Tables 3.10 and 3.11). This would explain why these cells are restored to normal upon transfusion, where a rapid regeneration of the ATP will take place (Valeri and Hirsch 1969) and hence a rapid reduction in the intracellular sodium ion will follow (Fortier et al 1969). On the other hand, if these cells are stored in vitro, under unsuitable circumstances, the sodium will continue to penetrate the cells, causing progressive swelling and ultimately lysis (Hurn 1968). It is known that storage of blood, in the liquid state, at $4^{o}C$ results in progressive depletion of its ATP content (Bartlett and Barnet 1960). This is probably due to its breakdown to provide the energy necessary for maintaining the active metabolism of the cell. Hydrolysis of ATP is normally accomplished by an enzyme called adenosine triphosphatase (ATP-ase) (Hurn 1968).

Unlike liquid storage, a reduction in ATP in the frozen-washed erythrocytes cannot be invoked as the basis of continued metabolism during storage because metabolic activity is completely inhibited at liquid nitrogen temperature. Therefore, this decrease must have occurred during the processes of thawing and washing when ATP-ase and metabolic activities are expected to be resumed. In fact an abnormal activity of the former in frozen-thawed erythrocytes has been observed by Rinfret in 1963, while Takehara and Rowe (1968) have related the magnitude of the ATP-ase activity in these cells to the rate of cooling used during freezing, the faster the cooling rate the higher the activation.

We have observed that the addition of 70 ml of ACD solution to the previously frozen-thawed-washed erythrocytes resulted in a substantial reduction in the rate of haemolysis of these cells on storage at $4^{\circ}C$. This was evidenced by the decrease in the free haemoglobin and potassium levels in the supernatant fluid, so that the blood will remain transfusable even after nine days of post-thaw storage. Earlier experiments have also shown that a solution of 5% albumin in isotonic saline has advantages over isotonic saline alone, as a resuspension medium, in reducing the rate of haemolysis during post-wash storage (Tables 4.1, 4.2, 4.12, 4.17 and 4.18 and Fig. 4.1 and 4.3). When ACD and 5% albumin were combined together in one solution the beneficial effect was summated and the results were even better than either medium alone. (Tables 4.5, 4.6, 4.7, 4.8, 4.12, 4.13, 4.21, 4.22, 4.23, 4.24 and Figure 4.1).

ACD might produce its effect through either, low pH (Maizles 1940) or citrate or because of its dextrose content. However, our

results show that neither the CPD solution, which contains the same molar concentration of citrate (Fig. 4.6), nor the low pH (Fig. 4.5) gave the same effect as observed with the addition of ACD to the resuspension medium. Also it is evident that glucose has no effect (Fig. 4.4a and 4.4b).

It was, thus, concluded that the beneficial effect of ACD in maintaining the post-thaw stability is due to two factors combined together, the low pH and citrate ions. When one of these factors is used alone the effect is less marked. The relatively low pH serves to maintain the ATP levels of the cells which is important in the ATP - dependent cation transport mechanism through the cell membrane which is, in turn, essential for prevention of red cell swelling. The enzymatic activity of the ATP-ase is pH-dependent with maximum activity occurring between pH 7.0 and 10.0 (Hurn 1968), and it is possible that decreasing the pH diminished the breakdown of ATP. Since, activation of ATP-ase is maximal after rapid freezing and thawing (Takehara and Rowe 1968), this would explain the higher recovery of intracellular ATP obtained with cells resuspended in ACD-saline, than those resuspended in saline alone (Table 4.26). This last table also shows that the recovered ATP levels after freezing, thawing, washing and resuspension in ACD-saline at pH 6.2 were 89.5% of the amount measured before freezing, while those of erythrocytes similarly treated but resuspended in saline alone were 65.9% (Fig. 3.11). This would also explain the early beneficial effect of ACD, even after four hours post-processing observed in all experiments.

Citrate is an anion which is incapable of penetrating the red cell and thus, will stop the sodium ions from penetrating the cell and hence help in preventing swelling and haemolysis.

Blood processed and reconstituted in this way has the following characteristics:

A. Haematological characteristics

1. A total intact cellular haemoglobin of about 50 g which is approximately 95% of the total haemoglobin donated.
2. Red cell haematological parameters all within normal limits.
3. Intact white blood cell content about 5-6% of the original leucocyte content prior to freezing. Most of the leucocytes recovered appeared to be of mononuclear origin and the majority of them are distorted.

B. Biochemical characteristics

On the day of processing and reconstitution to 0.5 Hct the final product has a supernatant free haemoglobin less than 0.2 g/unit and extracellular potassium of about 0.5 m mol/l (Tables 4.5 and 4.7). Whereas the intracellular potassium content of the recovered cells averages 7.8 m mol/10^{12}R.B.C. (Table 3.7). As regards the organic phosphate content, the recovered, reconstituted erythrocytes have about 75% of the pre-freeze value of 2,3 DPG and 90% of the ATP. Also, the pH of the blood is approximately 6.2.

When this blood is stored at 4°C for five days these levels are expected to change so that the supernatant haemoglobin would increase

to about 1.0 g/unit, supernatant K^+ to 6.5 m mol/l whilst the levels of the 2,3 DPG and ATP would decrease to 1.9, and 1.1 μ mol/g Hb respectively (Table 7.1). However, of utmost importance is that the blood is sterile during the whole period of storage.

C. In vivo survival

When the frozen-thawed-reconstituted red cells were transfused to the original donor on the day of processing, the 24 hours post transfusion ^{51}Cr survival was 95%. Also, the life span of the latter was normal (Table 5.2).

Furthermore, when the recovered-cells were stored at 4°C in ACD-saline medium for five days and then transfused to the same donor, the 24-hour- post-transfusion ^{51}Cr survival was 87% and again the life span was normal.

With these data in hand, we have started a frozen blood bank for clinical use according to the following protocol:

1. Blood used for freezing should be less than four days old.
2. Only group "O" Rh positive and group "O" Rh negative blood is frozen.
3. Frozen Blood should not be thawed before a minimum of period of storage of six months (maximum incubation period of serum hepatitis).
4. The thawed blood is processed automatically in the IBM 2991 Cell Processor. At the end of the last wash cycle 70 ml of ACD solution together with the same volume of isotonic saline are added to the washed cells and the mixture is thoroughly mixed.

5. We always keep a stock of nine units of frozen-washed reconstituted red cells in saline-ACD medium ready to be issued at any time and they are replaced after five days. Thus, the emergency requirement for frozen blood is covered day and night every day of the week.

In this way we have overcome the problem of the short shelf-life of the processed blood and achieved a better management of the frozen blood bank. At the same time the quality of the final product is as good or even better than standard liquid stored blood of seven days-old.

It might be argued that the low pH of that blood is a disadvantage, first because, at this pH the 2,3 DPG content is not well maintained, and second, the transfusion of such blood might be harmful. However, it has to be remembered that ordinary blood collected in ACD and stored at $4^{\circ}C$ for three weeks has a pH as low as 6.4 or 6.5 (Bunker 1966). Such blood has been used successfully for many years and is still in common use by many centres without untoward effects. This might be due to the fact that the added acids in the blood are usually buffered by the plasma bicarbonate and red cell haemoglobin before transfusion, and thus does not represent the same danger caused by transfusion of unbuffered acids. Transfusion of blood in which the normal buffers have been diminished might result in lowering of the recipient's blood pH as a result of dilution of his plasma bicarbonate. However, it seems that the resultant acidosis is mild and very transient and it is rapidly corrected by the addition of new bicarbonate produced by the metabolism of citrate and lactate in the transfused

blood (Bunker 1966). Since ACD contains large concentrations of sodium, and because the bicarbonate is produced in large amounts, such a transfusion may give a metabolic alkalosis. Bunker also maintained that even when large volumes of blood are transfused rapidly so that the recipient's plasma bicarbonates is diluted to half its normal concentration, the blood pH of the patient would only fall to 7.25 or 7.3, and this mild decrease would only last for a short period. When we come to frozen blood, we found that despite the fact that this blood is free of plasma, and thus it contains no bicarbonate, it still has the high buffering capacity of 50 grams of haemoglobin. In addition a dilution of the recipient's plasma bicarbonate, (providing it is normal), to half its value would result only after the rapid transfusion of six or eight units of frozen blood, such a condition is hardly ever likely to be required in medical cases. Furthermore if large volumes of frozen blood are to be transfused arrangements could be made to resuspend this blood in isotonic saline alone and transfuse the blood within 24 hours of processing.

As regards the 2,3 DPG, we know that this compound is important in the role it plays in the transportation of oxygen. Thus, it is known that the oxygen affinity of haemoglobin is rapidly increased in the blood during its storage in ACD at $4^{\circ}C$ (Valtis and Kennedy 1954). This was found to be positively correlated with the observed decrease in the red cell 2,3 DPG content (Benesch and Benesch 1967, Chanutin and Curnish 1967). Thus patients who receive large volumes of blood stored in ACD for more than seven days are expected to have an increase in the haemoglobin affinity for oxygen in their blood. For this reason, it has been suggested that the latter blood is

undesirable and that only blood with high levels of 2,3 DPG should be transfused. Such a view, if adopted by many physicians would seriously compromise blood bank management everywhere.

It seems likely that the importance of 2,3 DPG in transfusion has been overstressed, for, although there is no doubt that the above mentioned changes in the oxygen affinity of the recipient take place, their pathological effects on diseased patients have not been defined. On the other hand we know from the vast experience everywhere that massive transfusion up to complete volume replacement with stored blood does not compromise the delivery of oxygen to the tissues as evidenced by the high rate of survival of those patients (Beutler et al 1974). This is probably due to the fact that rapid restoration of the depleted 2,3 DPG takes place immediately after transfusion (Valeri and Hirsch 1969, Fortier et al 1969).

Finally it has to be emphasized that the low pH of the frozen blood resuspended in ACD-saline medium is important for maintaining the ATP content of the red cells, which is in turn an important factor in determining the in vivo erythrocyte survival. We believe that this is more important than the 2,3 DPG level, because cells that are capable of surviving after transfusion would have the opportunity to resynthesize a normal content of 2,3 DPG.

IN VIVO SURVIVAL OF FROZEN BLOOD

As mentioned in the introductory section estimation of the post-transfusion survival of frozen-thawed red blood cells by the radioactive chromium method was based on the assumption that labelling of the cells by ^{51}Cr is not affected by the various processes involved in the preparation of such cells. The reason for choosing autologous transfusion studies have also been mentioned before; in short, there are difficulties in estimating accurately the plasma volume in diseased patients and eliminating immunological factors which might affect the in vivo survival. We preferred to transfuse the whole unit of blood in order to simulate what really occurs in practice. The 100% chromium activity, in our study, represents the real zero time recovery. We preferred this method of calculation rather than that of extrapolating backwards from the chromium count at 30 minutes or one hour to obtain a hypothetical zero time count as the latter method assumes that there is no rapid non-linear loss of labelled but damaged cells of the type which we were trying to measure.

The addition of the radioactive chromium post-thaw offers the advantage that excess radioactivity, if present, is washed out during routine processing and thus no extra washing is required which might produce unnecessary damage.

The mean 24-hour post-transfusion survival of erythrocytes that were frozen-thawed and processed by manual batch washing on the day of processing was 93.4% of the transfused cells. Since the in vitro recovery of those cells was about 92.5%, it is thus concluded that approximately 86% of the original cells used for freezing were still viable in the recipient's circulation 24 hours after transfusion.

Valeri and Runck (1969a) have used the term therapeutic effectiveness to describe the latter percentage which was computed by multiplying the in vitro recovery (%) by the 24-hour chromium survival (%).

Red blood cells frozen, stored, thawed and processed by the IBM Automatic Cell Processor then transfused to the original donor on the same day of processing had a mean 24-hour post-transfusion survival of 95.2%. However, the latter was not statistically different from that of the manually processed erythrocytes.

Similarly the therapeutic effectiveness of the automatic method of processing was computed and found to be approximately 90%. When the survival of these cells was followed for four successive weeks, it was found that they have a normal $T\frac{1}{2}$ ^{51}Cr which indicates a normal long-term survival. This confirms the observation of Jones et al (1957) and Valeri and Runck (1969a) that red cells surviving the first 24-hours after transfusion would continue for a normal life span when transfused to the original donor who was a healthy individual.

The observation that frozen-processed-chromium labelled red cells have a normal $T\frac{1}{2}$ ^{51}Cr suggested that the chromium elution from these cells was not affected by the various processes involved in their preparation.

Since saline-ACD was the medium of choice recommended for reconstitution and post-thaw storage of the frozen-thawed deglycerolized red cells, it was necessary to study the in vivo survival of erythrocytes resuspended and stored for five days in such medium. The survival of these cells was measured in ten cases of auto-

transfusion utilizing the ^{51}Cr - ^{125}I technique. The mean 24-hour post-transfusion survival was 87% ± 5.7 which indicates a therapeutic effectiveness of this method of preservation of about 82%. Also the $T\frac{1}{2}$ ^{51}Cr was determined in five of those cases and was found to be about 23 days which indicates a normal survival.

Following transfusion, the recipients' plasma haemoglobin rises to levels that depend on the amount of supernatant haemoglobin and the percentage of non viable (irreversibly damaged) red cells in the transfused blood as well as on the state of the reticuloendothelial system, the level of the haptoglobin of the recipient and the rate of transfusion. The highest level of haemoglobinaemia was observed 30 minutes following transfusion after which the recipients' plasma haemoglobin delcined but was still higher than the pretransfusion level even after 24-hours.

The pre-transfusion levels of the plasma haemoglobin in the cases studied ranged from 0.002 g/l to 0.22 g/l with a mean of 0.05 ± 0.05 g/l. This value was higher than the values reported by Dacie & Lewis 1970. This is most probably due to the artificially produced haemolysis during blood sampling and separation of the plasma.

Units of frozen-saline resuspended blood that were transfused on the same day of processing (Table 5.2) had a free supernatant haemoglobin ranging from 0.263 g/unit to 2.387 g/unit with a mean value of 1.33 g/unit. However, in two of these units (C.D. and J.D.C.) the supernatant was separated from blood that was left in the giving set after transfusion overnight at room temperature and thus the free haemoglobin levels was higher than that transfused. This is also

evident from the wide difference between the expected and observed values of the recipient's plasma haemoglobin in these two cases when compared with the others.

Units of frozen erythrocytes resuspended in saline-ACD for five days and then transfused showed a supernatant free haemoglobin levels ranging from 0.268 to 1.243 g/unit, with a mean of 0.750 g/unit on the day of transfusion.

It was expected that the highest haemoglobin levels in the recipient's plasma would occur at the 15 minute post-transfusion sample, assuming that complete mixing would have occurred and that non viable cells would have been removed within this time. However, the observation that the plasma haemoglobin in the 15 minute sample was lower than that expected suggests that clearance of the haemoglobin was rapid and started immediately. The increase of the plasma haemoglobin observed in the 30 minute sample, thus perhaps reflects the destruction and removal of irreversibly damaged cells.

With exception of three cases (G.S., J.D.C. and K.A.A.) out of 14, there was no unexpected haemoglobinaemia after the transfusion of either zero or five day stored blood. Even in these three exceptions, the differences between the expected and the observed levels of plasma haemoglobin were not so great that they could possibly be due to technical errors. Also in none of the cases was there an increase in the recipient's plasma radioactivity over that observed prior to transfusion.

The absence of overt haemoglobinuria together with the gradual fall in the recipient's plasma haemoglobin level suggests that all

the haemoglobin in the circulation was combined with the haptoglobin and was efficiently cleared by the reticulo-endothelial system.

Because of the low content of the frozen blood leucocytes and platelets, there might be some concern about the effect of transfusion of large volumes of such blood on the levels of these cells in the recipient circulation. In this study although only one unit of blood was transfused at a time, the results show that even after auto-transfusion, a single unit of frozen blood can provoke a reactionary leucocytosis and thrombocytosis. This observation has also been reported by Haynes et al (1962) and Murray et al (1962). The leucocytosis started immediately after the transfusion and reached a maximum after 30 minutes, whereas the thrombocytosis was observed up to 24 hours later. Differential counts in the recipient's white blood cells showed that the increase in leucocytes was mainly due to neutrophil leucocytosis.

LEUCOCYTE CONTENT OF THE FROZEN BLOOD

Having satisfied ourselves that blood could be produced with, a shelf-life of up to five days and with a satisfactory biochemical and bacteriological status, post-thaw, we investigated a further area where little data has been published. One of the major advantages of frozen blood is its documented reduction in cases of febrile transfusion reactions involving leucocyte antibodies. However, practically nothing is known about the residual leucocyte material which might be transfused. We have, therefore, investigated

this problem in several different ways.

In the preliminary studies leucocyte counts were performed both electronically using a Coulter Counter Model S and microscopically in a haemocytometer using the method of Dacie and Lewis (1970). Counts from both methods were found to be comparable. Sometimes the electronic counts were higher than the visual ones, this is possibly because some other cell fragments were counted as intact white cells. However, because the electronic method of leucocyte counting is a standard method for calculation of the white cell content in HL-A-poor blood elsewhere (Halterman et al 1972, Helgeson et al 1973, Miller et al 1973, Tenczar 1973, Halterman et al 1973, Perkins et al 1973, Polesky et al 1973, Meryman and Hornblower 1973), it has been adopted as the sole method throughout the rest of this work. The leucocyte content of the blood was expressed as million per gram of haemoglobin. We preferred this method of computing the actual number of white cells present in the blood rather than calculations based on the blood volume as the latter might be misleading. The processes of glycerolization, freezing, thawing and washing appears to be destructive to the white cells. Throughout this study we systematically obtained a reduction in the white cell count, due to the above mentioned processes, ranging from 93.7 to 95% of the original white cell content prior to freezing. Smears prepared from leucocytes after separation of recovered cells with dextran or hydroxyethyl starch, stained with May-Grünwald - Giemsa's stain, revealed the presence of only mononuclear cells, particularly lymphocytes. However the majority of these cells appeared distorted. This implies that white cells other than the lymphocytes are too delicate to

stand the processes of glycerolization, rapid freezing, thawing and washing. These results agree with those obtained by Meryman and Hornblower (1973) who separated leucocytes from a unit of whole blood and resuspended them in human plasma. After glycerolization, freezing, thawing and washing in a continuous-flow system, only 6% of the original leucocytes were recovered intact, 99% of which were lymphocytes. Our results also agree with those published recently by Crowley and Valeri (1974) who, though claiming a higher percentage of polymorphnuclear cell recovery, have also observed a preferential removal of polymorphnuclear leucocytes by the process of glycerolization, freezing, thawing and washing.

Although a frozen red cell suspension containing 5% of the normal level of white cells might be considered as a satisfactory leucocyte-poor preparation, the mechanism of removal of these cells was not known and this threw some doubt on the acceptability of this blood as an ideal preparation, because the blood might still contain some HL-A Antigen in the form of leucocyte and platelet debris. Crowley and Valeri (1974) suggested that the process of glycerolization damages the white cells and that these damaged cells agglomerate into clot like masses which are removed during the washing procedure. On the other hand Meryman and Hornblower (1973) suggested that removal of the white cells is not the result of washing them out of the red cell suspension, but due to their aggregation into mucous-like masses that stick to the wall of the processing chamber in all washing systems. Despite this controversy, the implication is the same, namely the blood will contain only a fraction of its original leucocytes in the form of intact cells whilst the damaged cells are

aggregated and either removed in the wash solution or adhere to the wall of the processing bag.

Experiments with labelled leucocytes however, have clearly shown that blood frozen by the low glycerol-rapid-freezing technique, thawed and washed in the IBM 2991 Automatic Cell Processor, though containing only 5-6% of intact white cells, still has a high level of input material in the form of leucocyte debris. However this debris could be reduced on transfusion by passing the blood through a Swank-Transfusion Filter. A more effective method of removing the leucocyte debris from the processed blood, however, was to remove the buffy coat automatically after each wash step. It is unfortunate, however, that the latter technique, though most effective, results in the loss of a further 20% of the red cells.

Experiments with labelled lymphocytes showed that only about one third of the labelled material was washed out of the red cell suspension by the wash solutions. Approximately 25% remained in the processing bag in the form of gelatinous masses sticking to the wall of the bag. The demonstration that as much as 40% of the labelled material was remaining in the final red cell suspension was most surprising. Since the normal leucocyte recovery in our product, as determined by the conventional methods was 5-6% of intact cells, then the major part of this material was in the form cellular debris. A significant amount of this debris could be removed by either filtration through a Swank-Transfusion Filter or by removing the buffy coat at the last wash step. In the latter case the reduction was obviously (Table 6.1b) due to an increase in the amount of debris

removed by the wash solutions, for the amount of radioactivity remaining in the bag was almost the same as that in Table 6.1a .

Maximum removal of the leucocyte derived material was achieved by removing the buffy coat after each wash step, however this was at the expense of losing a higher percentage of red cells. In this case the majority of the leucocyte material was washed out in the waste and a minimum amount remained in the processing bag. This suggests that, if the leucocyte debris are given sufficient time in contact with each other some of them will coalesce into gelatinous masses and settle to the bottom where they stick to the wall of the bag (Table 6.1a and 6.1b) otherwise they remain separate on the surface and liable to be removed as a buffy coat.

What applies for the lymphocytes applies also to the granulocytes in this respect. However, as regards the platelets only 2% of the labelled material remained in the final product, while the rest was removed with the wash solutions. The amount of platelets remaining seems to be highly dispersed in the blood as very fine particles as they could not be removed with either filtration or by the technique of buffy coat removal.

When the viability of the remaining cells was tested with the dye exclusion test, none of the cells examined appeared viable. However when these cells were stimulated by PHA in a tissue culture media, some of them showed a weak response. When the viability in the latter condition was expressed as the activity of tritiated thymidine incorporated by one million lymphocytes and the results were compared to that of lymphocytes from the same blood prior to freezing we found that the ratio was 13%. However, it has to be

noted that in order to separate the number of lymphocytes which are sufficient to set up one test we had to use a whole unit of frozen blood, whilst in case of the fresh blood 20 ml would fulfil the requirements. Taking these two figures together would indicate that the ability to respond to PHA is reduced 200 fold in frozen blood compared to fresh blood.

The conflict between both results of assaying the viability of the separated frozen lymphocytes can probably only be explained on the basis of the sensitivity of each test.

The finding that some lymphocytes separated from frozen blood are viable is not surprising, both Meryman and Hornblower (1973) and Crowley and Valeri (1974) have also reported the presence of viable lymphocytes in their preparations.

However, although frozen blood contains some of the HL-A antigen containing material in the form of intact lymphocytes and fragments, and although a few of these cells are viable, yet they are not antigenic to homologous lymphocytes when incubated with them in the one way mixed lymphocyte culture.

This is probably due to some changes occurring in the cell membrane, and which might have led to loss of the antigen sites, as a result of the various processes involved in preparation of the blood. Such changes are known to occur after freezing and thawing Meryman and Hornblower (1973).

However, such speculation does not agree with the results of testing the antigenicity of frozen blood in laboratory animals.

In vivo assessment of antigenicity in rabbits showed that frozen blood does contain some human antigens in the form of intact lymphocytes and cell fragments able to stimulate the production of lymphocyte antibodies. However, the amount of this antigen seems to be lower than that present in the fresh blood. Thus it might be possible that freezing and thawing produces changes in the antigen sites of the lymphocytes to the extent that these cells would not be recognised by third party human lymphocytes in vitro, but could be identified by the intact immune mechanism of the rabbit when injected in vivo. A significant fraction of this antigen can be removed on transfusion by passing the blood through Swank Transfusion Filters which probably removes large and medium sized (40 μ) leucocyte aggregates. Naturally, what occurs in rabbits does not necessarily apply to human beings and studies in man are required in order to confirm whether or not frozen blood is antigenic and if so, what is the minimal dose of antigen required to sensitize a recipient. A retrospective study in one multiple transfused patient who experienced a non haemolytic reaction with every transfusion of fresh blood, showed that frozen non-filtered blood is antigenic and might cause a febrile non haemolytic reaction if more than one unit of the blood transfused shared a common leucocyte antigen to which the

recipient was previously immunized (Pepper 1974 unpublished work).

In another recipient, who was apparently not immunized and received only frozen blood, lymphocyte antibodies were detected in his serum after multiple transfusion of frozen-non-filtered blood (Pepper 1975 unpublished work).

It was, therefore, evident that frozen blood is immunogenic to laboratory animals as well as to human beings. However, transfusion of frozen blood processed in the way recommended in this study, (with the buffy coat removed) was highly successful in prevention of leucocyte - transfusion reaction and no incidence of such a reaction was reported (apart from the above two cases) during the transfusion of more than one thousand units. Whether or not this blood is suitable for prospective transplantation candidates is not yet settled. We know that frozen blood, is to date, the product with the lowest HL-A antigen content and therefore it is expected to be the most suitable preparation for prospective transplant recipients. On the other hand there is some recent speculation about the necessity for some HL-A antigen in the transfused blood for a renal transplant operation to be successful (Opelz and Terasaki 1974). The last named authors based their conclusions on retrospective studies of the rate of one year renal graft survival in several centres and concluded that a certain amount of antigen, capable of producing immuno-tolerance but short of producing HL-A cytotoxic antibodies is desirable in the blood to be transfused to those patients before the transplant operations.

Whether or not this view is correct is a matter for speculation,

and if correct will require a planned study to determine the amount of antigen necessary for immunization of a non-sensitized recipient as well as the amount needed to produce tolerance.

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